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BIOLOGICAL BULLETIN

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THE BIOLOGICAL BULLETIN accepts original research reports of intermediate length on a variety of subjects of biological interest. In general, these papers are either of particular interest to workers at the Marine Biological Laboratory, or of outstanding general significance to a large number of biologists throughout the world. Normally, review papers (except those written at the specific invitation of the Editorial Board), very short papers (less than five printed pages), preliminary notes, and papers which describe only a new technique or method without presenting substantial quantities of data resulting from the use of the new method cannot be accepted for publication. A paper will usually appear within four months of the date of its acceptance.

The Editorial Board requests that manuscripts conform to the requirements set below; those manuscripts which do not conform will be returned to authors for correction before review by the Board.

1. ***Manuscripts.*** Manuscripts must be typed in double spacing (*including* figure legends, foot-notes, bibliography, etc.) on one side of 16- or 20-lb. bond paper, 8½ by 11 inches. They should be carefully proof-read before being submitted and all typographical errors corrected legibly in black ink. Pages should be numbered. A left-hand margin of at least 1½ inches should be allowed.

2. ***Tables, Foot-Notes, Figure Legends, etc.*** Tables should be typed on separate sheets and placed in correct sequence in the text. Because of the high cost of setting such material in type, authors are earnestly requested to limit tabular material as much as possible. Similarly, foot-notes to tables should be avoided wherever possible. If they are essential, they should be indicated by asterisks, daggers, etc., rather than by numbers. Foot-notes are not normally permitted in the body of the text. Such material should be incorporated into the text where appropriate. Explanations of figures should be typed double-spaced and placed on separate sheets at the end of the paper.

3. ***A condensed title*** or running head of no more than 35 letters and spaces should be included.

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THE MARINE BIOLOGICAL LABORATORY

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II. ACT OF CORPORATION

No. 3170

COMMONWEALTH OF MASSACHUSETTS

Be it Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips, and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL]

HENRY B. PIERCE,
Secretary of the Commonwealth

III. BYLAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

(Revised February 11, 1972)

I. The members of the corporation shall consist of persons elected by the Board of Trustees.

II. The officers of the Corporation shall consist of a Chairman of the Board of Trustees, President, Director, Treasurer and Clerk.

III. The Annual Meeting of the members shall be held on the Friday following the second Tuesday in August in each year at the Laboratory in Woods Hole, Massachusetts, at 9:30 A.M., and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and nine Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the members may be called by the Trustees to be held at such time and place as may be designated.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

VI. Inasmuch as the time and place of the Annual Meeting of members are fixed by these bylaws, no notice of the Annual Meeting need be given. Notice of any special meeting of members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least (15) days before such meeting, to each member at his or her address as shown on the records of the Corporation.

VII. The Annual Meeting of the Trustees shall be held promptly after the Annual Meeting of the Corporation at the Laboratory in Woods Hole, Massachusetts. Special meetings of the Trustees shall be called by the Chairman, the President, or by any seven Trustees, to be held at such time and place as may be designated, and the Secretary shall give notice thereof by written or printed notice, mailed to each Trustee at his address as shown on the records of the Corporation, at least one (1) week before the meeting. At such special meeting only matters stated in the notice shall be considered. Seven Trustees of those eligible to vote shall constitute a quorum for the transaction of business at any meeting.

VIII. There shall be three groups of Trustees:

(A) Thirty-six Trustees chosen by the Corporation, divided into four classes, each to serve four years. After having served two consecutive terms of four years each, Trustees are ineligible for re-election until a year has elapsed.

(B) Trustees *ex officio*, who shall be the Chairman, the President, the Director, the Treasurer, and the Clerk.

(C) Trustees *Emeriti*, who shall be elected from present or former Trustees by the Corporation. Any member of the Corporation in good standing who has attained the age of seventy years, or has attained the age of sixty-five and has retired from his home institution, and who has served a full elected term as a regular Trustee, shall be designated Trustee Emeritus for life at the next annual meeting provided he signifies his wish to serve the Laboratory in that capacity. Any regular trustee who qualifies for emeritus status shall continue to serve as Trustee until the next Annual Meeting whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation. The Trustees *ex officio* and *Emeriti* shall have all the rights of the Trustees, except that *Trustees Emeriti* shall not have the right to vote.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

IX. The Trustees shall have the control and management of the affairs of the Corporation. They shall elect a Chairman of the Board of Trustees who shall be elected annually and shall serve until his successor is selected and qualified and who shall also preside at meetings of the Corporation. They shall elect a President of the Corporation who shall also be the Vice Chairman of the Board of Trustees and Vice Chairman of meetings of the Corporation, and who shall be elected annually and shall serve until his successor is selected and qualified. They shall appoint a Director of the Laboratory for a term not to exceed five years, provided the term shall not exceed one year if the candidate has attained the age of 65 years prior to the date of the appointment. They may choose such other officers and agents as they may think best. They may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them except those chosen by the members, at any time. They may fill vacancies occurring in any manner in their own number or in any of the officers. The Board of Trustees shall have the power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

X. The Associates of the Marine Biological Laboratory shall be an unincorporated group of persons (including associations and corporations) interested in the Laboratory and shall be organized and operated under the general supervision and authority of the Trustees.

XI. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

XII. The account of the Treasurer shall be audited annually by a certified public accountant.

XIII. These bylaws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the bylaws will be acted upon.

RESOLUTIONS ADOPTED BY THE TRUSTEES:

I. RESOLVED:

(A) The Executive Committee is hereby designated to consist of not more than ten members, including the *ex officio* members (Chairman of the Board of Trustees, President, Director and Treasurer); and six additional Trustees, two of whom shall be elected by the Board of Trustees each year, to serve for a three-year term. (August 11, 1967.)

(B) The Chairman of the Board of Trustees shall act as Chairman of the Executive Committee, and the President as Vice Chairman. A majority of the members of the Executive Committee shall constitute a quorum and a majority of those present at any properly held meeting shall determine its action. It shall meet at such times and places and upon such notice and appoint such sub-committees as the Committee shall determine. (August 12, 1966).

(C) The Executive Committee shall have and may exercise all the powers of the Board during the intervals between meetings of the Board of Trustees except those powers specifically withheld from time to time by the Board or by law. (August 16, 1963).

(D) The Executive Committee shall keep appropriate minutes of its meetings, and its action shall be reported to the Board of Trustees. (August 16, 1963).

II. RESOLVED:

The elected members of the Executive Committee be constituted as a standing "Committee for the Nominations of Officers," responsible for making nominations, at each Annual Meeting of the Corporation, and of the Board of Trustees, for candidates to fill each office as the respective terms of office expire (Chairman of the Board, President, Director, Treasurer, and Clerk). (August 16, 1963).

III. RESOLVED:

Any member of the Corporation in good standing who has attained the age of seventy years, or has attained the age of sixty-five and has retired from his home institution, shall automatically be designated a Life Member of the Corporation provided he signifies his wish to retain his membership in the Corporation. Life Members shall not have the right to vote and shall not be subject to the payment of any dues. (February 16, 1973).

IV. REPORT OF THE DIRECTOR

To: The Trustees of the Marine Biological Laboratory

Speaking on the heritage of excellence at another famous institution, during the celebration of the one hundredth anniversary of the birth of William H. Welch, Alan Gregg said, "Something extraordinarily precious comes out of the close but entirely free association of men and women of superior character and capacity, because it lasts so long and because it never comes from or appeals to mediocrities." He could have been describing the Marine Biological Laboratory.

It is one of the Director's responsibilities to convey the spirit of the Laboratory not only to fellow scientists, but to foundations and the public at large. It is a formidable task. The greatness of the Laboratory cannot be assayed in a phrase. Now in its 87th year of service to world science, the Laboratory's research and justly renowned summer courses have traditionally been—and remain—at the cutting edges of their respective fields.

We are justly proud of the twenty-nine winners of the Nobel Prize who have conducted various aspects of their work at the Laboratory, and of the nearly sixty members of the National Academy of Sciences who are also members of our Corporation (about ten per cent of the Corporation's membership). However a roster of distinguished investigators tells only a part of the story. The life blood of the Laboratory is the student and beginning investigator, who finds in the unique scientific community of Woods Hole an exceptionally favorable environment for growth and maturation. The Laboratory's greatness lies, in large part, in its insistence that teaching and research are inextricably interwoven.

Despite the inroads of inflation into our already meager resources, we have been firm in our resolve to increase the vitality of the Laboratory during the winter months. The appointment of E. F. MacNichol, Jr. as Assistant Director and his immediate and effective grasp of many problems of equipment and research services has enabled the Director to devote an increasing amount of his own time to fund-raising. During the year some two dozen foundations were visited; at least a dozen proposals have been submitted, with others in the offing.

The Henry L. and Grace Doherty Charitable Foundation has pledged $500,000 toward the establishment of an Ecosystems Center at the Laboratory. However the grant is contingent upon our securing additional funds to support the Center. Of the $2.5 million dollars needed to underwrite the Center for the first five years, over a quarter of that amount has been pledged. In addition to the Doherty commitment the Clowes Family Foundation has given and pledged $40,000 per year for at least four years and the Grass Foundation an additional $40,000. I am also pleased to report that the Rowland Foundation will provide $110,000 in 1974 and 1975 in support of MacNichol's Laboratory of Sensory Physiology bringing the total amount for year-round programs to over $800,000. Securing the remaining funds is one of the priorities of our major fund raising campaign.

In this year's efforts I have relied heavily on some of the "at large" officers and Trustees. Our Chairman, Denis Robinson and Treasurer, Mr. A. T. Daignault, and indeed all four at large Trustees have responded frequently to requests for assistance. Their location in New York, the seat of many of the foundations, has made Mr. Daignault and Mr. Golden especially vulnerable to the Director's calls. It is against this background that I observe that the Trustees have moved to reapportion the composition of future classes. The Laboratory has thirty-six active Board members representing four classes of nine Trustees each. One new class is elected by Members of the Corporation at the annual meeting. In the past each class has included eight biologists elected by the Corporation and one "at large" member proposed by the Executive Committee and approved by the Corporation. In view of the shifting demands being placed on Trustees and the increasing need for our Board to represent a broader segment of the business and philanthropic population, it was felt by the Board that this ratio should be changed. Beginning in 1974 the Corporation Members will elect six biologist Trustees and approve the election of three at large Trustees by the Executive Committee. The first election under this new arrangement will take place at the 1974 Annual Meeting.

"*Education for uncertainty*"

We are passionately dedicated to the principle that the main hope for progress against disease and the deterioration of the environment lies in research to which the student should have early exposure. The critical turn of mind fostered by research tempers the student's approach to the evolving base of science and its applications. It forms the basis of what John Whitehorn termed "*education for uncertainty.*"

In the past few years there has been a phasing out of federal grants for training programs. However, at least in 1974 the Laboratory is still receiving federal support for four of its summer courses and training programs. The National Institute of Child Health and Human Development is funding the Research Program in Reproductive Biology and the Embryology course. Dr. Eric Davidson heads the Embryology course and Dr. Robin A. Wallace is coordinator of the Research Program in Reproductive Biology.

The National Institute of Neurological Diseases and Stroke has announced it will restore its support of the Frontiers in Research and Training Program. The Frontiers Program was initiated in the summer of 1971 on an experimental basis through support from the Institute. In 1973 the program suffered a serious cutback in funds.

The Frontiers Program is designed to introduce increasing numbers of well-qualified scientists of minority ethnic groups into the neurosciences. It is an advanced training program primarily for individuals at the postdoctoral level who are seeking further experience in research or new ways of strengthening their capacity as instructors of neuroscience. The Program Coordinator is Dr. James G. Townsel.

The National Science Foundation has announced its intention to support the Laboratory's new program in History of Evolutionary Biology on a one-summer trial basis in 1974. Dr. William Coleman of Johns Hopkins will head this summer's program which will focus on the development of evolutionary doctrine from the late 18th to the early 20th century.

Support for three summer programs will come from private foundations and individuals. The Grass Foundation continues to underwrite the Neurobiology course and the Waksman Foundation will support the Marine Ecology course. We expect Dr. Ernest B. Wright to continue his generous contribution to the Research Training Program in Excitable Membrane Biophysics and Physiology, to which IBM is also contributing.

During the month of January, 28 undergraduate students spent the "short-term" or "mini-semester," as the period between the regular academic semesters is often called, studying developmental biology at the Laboratory. The students came from a wide variety of institutions that included the University of Wyoming, Texas A & I, Johns Hopkins University and Smith and Mount Holyoke Colleges.

Dr. Louis E. DeLanney of Ithaca College was the coordinating instructor in cooperation with the Director. The teaching staff included Dr. David Walters of Boston University, Ms. Joanne Fortune, a laboratory instructor from Cornell, Doctors John and Annette Coleman from Brown University and Doctors Lester and Lucena Barth, resident scientific investigators at the MBL.

During the students' four-week stay they resided in the MBL dormitory. Their schedule included two morning lectures five days a week, formal opportunities to use the laboratories in the afternoon and informal day and night laboratory sessions with Dr. DeLanney and Ms. Fortune.

In addition to the scheduled programs there were 18 guest seminars which gave the students an opportunity to see how specialists in their field approach and resolve scientific problems. Investigators at the MBL and the Oceanographic Institution were among the speakers at the seminar.

The Oceanographic Institution gave the students an afternoon tour of its facilities and an opportunity to go aboard the KNORR and observe how a vessel can be used for seagoing research. The students also went out on the Laboratory's research vessel, the A. E. VERRILL, and participated in collecting and towing.

Each student paid $580 to cover laboratory expenses and room and board. The students may receive four semester hours of credit at their own college for their intensive course work.

Plans are under way to expand the program in January, 1975, to include more students and to offer a greater selection of courses. Already scheduled in addition to Developmental Biology is a Neurobiology course from the joint MBL and the Boston University Marine Program, which will be taught by Doctors MacNichol, Fred Lang and Alan Fein. A grant made jointly to Boston University and the Laboratory by the Alfred P. Sloan Foundation will provide funds for equipment and scholarships.

Thus we continue to attempt, in John Shaw Billings' words, "To give to the world men who cannot only sail by the old charts, but who can make new and better ones for the use of others."

Losses—and gains

As 1973 drew to a close, Mr. Robert Kahler retired as Superintendent of Buildings and Grounds, after forty-seven years' service, twenty-five of them as Superintendent. His length of service is impressive, of course, but the numbers tell only a small part of the story of his effective and unflagging service to the Laboratory.

Mr. Robert Gunning has been named to succeed Mr. Kahler.

Dr. Arnold Lazarow, Professor and Chairman of the Department of Anatomy at the University of Minnesota, received the highest honor of the American Diabetes Association at its twenty-third annual meeting in Chicago. He was the recipient of the Association's Banting Medal and delivered the annual Banting Memorial Lecture.

Seven members of the Corporation were elected to the National Academy of Sciences in 1974: Drs. Martin Gibbs, a member of the staff of the Research Program in Experimental Marine Botany; Jerome Gross; Jerard Hurwitz; Alex Novikoff; Ladd Prosser, Trustee (Class of 1976); Sarah Ratner and DeWitt Stetten. Election to membership in the NAS is considered to be one of the highest honors that can be accorded to an American scientist. The fact that about ten per cent of the Corporation's membership has been so honored (over five per cent of the total membership of the Academy in all fields are MBL biologists) continues to point to the Laboratory as one of the Nation's leading centers of research and communication in the life sciences.

1. MEMORIAL

ELMER G. BUTLER

BY MALCOLM S. STEINBERG

All of his many friends at the Marine Biological Laboratory are saddened at the death of Elmer G. Butler, who passed away on February 23, 1972, after a brief illness. Born February 13, 1900, he graduated from Syracuse University and received the Ph.D. from Princeton University in 1926, where he shortly joined the faculty. Within eight years he rose to the chairmanship of the Department of Biology, a post he held until 1948.

A native of Parish, New York, Elmer Butler was well known for his contributions to the study of regeneration in amphibian extremeties. His interest focused on the role of nerves in regeneration, and his last experiments dealt with the regeneration of extirpated segments of the spinal cord itself.

Active in many professional organizations, Dr. Butler was a Past President of AIBS, the American Society of Zoologists, and the Society for the Study of Development and Growth (now the Society for Developmental Biology). He had served as Chairman of N.I.H.'s Cell Biology Study Section and in editorial capacities for the Journal of Morphology, Developmental Biology, and the Journal of Experimental Zoology.

A member of the Corporation of the Marine Biological Laboratory since 1945, Elmer Butler served at various times on the Evening Lecture Committee, the Library Committee, the Buildings and Grounds Committee and the Nominations Committee. He was elected to four terms as Trustee (1951-55, 1955–59, 1960–64, 1964–68) and to three terms on the Executive Committee of the Board of Trustees (1952–54, 1956-58, and 1965–67). In all of these capacities he lent his council, always willingly and always in a quiet manner.

A generous and congenial man, it was characteristic of Butler's gentle humor for him to turn to a friend during the 1968 Annual Meeting of the Corporation, after Director H. Burr Steinbach's announcement of his election to the office of Trustee Emeritus, and add with a broad smile, "an honor long overdue"!

Eleanor and Elmer Butler had no children of their own, but one by one "adopted" Elmer's graduate students, all of whom retained and returned this devotion forever thereafter.

We join in extending to Eleanor our deepest sympathy.

2. The Staff

EMBRYOLOGY

I. Instructors

Eric Davidson, Associate Professor of Biology, California Institute of Technology, Director of course

Gary Freeman, Associate Professor of Biology, University of Texas, Associate Director of course

Fotis Kafatos, Professor of Biology, Harvard University

L. Dennis Smith, Associate Professor of Biology, Purdue University

Igor Dawid, Staff Member, Carnegie Institution of Washington, Department of Embryology

Joseph Gall, Professor of Biology, Yale University

II. Special Lecturers

Joseph Ilan, Case Western Reserve University

Everett Anderson, Harvard University

Paul Schroeder, Washington State University

Richard Halberg, Cornell University

Paul Gross, University of Rochester

Fred Wilt, University of California, Berkeley

Barbara Hamkalo, University of California, Irvine

William Telfer, University of Pennsylvania

Robert Briggs, University of Indiana

Burke Judd, University of Texas, Austin

David Epel, Scripps Institution of Oceanography

Walter Gehring, Yale University

Peter Wellauer, Carnegie Institution of Washington

III. Technical Assistant

Christopher Amenson, Graduate Fellow, California Institute of Technology

IV. Laboratory Assistants

Gregory Britten, Marine Biological Laboratory
Barbara Ehrlich, Brown University

V. Lectures

Gary Freeman	Is oogenesis necessary for subsequent development?
Joe Gall	Chromosome activity during oogenesis
Eric Davidson	Synthesis and storage of RNA during oogenesis
Fotis Kafatos	Specific protein synthesis during chorion formation in insects
Dennis Smith	Protein synthesis during oogenesis and egg maturation
Igor Dawid	Mitochondrial development during oogenesis
Everett Anderson	Comparative ultrastructure of the female gamete
Paul Schroeder	Endocrine control of oocyte growth in annelids
Richard Halberg	Specific protein synthesis during oogenesis in *Xenopus*
Paul Gross	The synthetic organization of the sea urchin egg
Fred Wilt	Cytoplasmic polyadenylation of maternal RNA following activation of sea urchin eggs
Barbara Hamkalo	Visualization of transcription in oocytes
William Telfer	Nurse cell function during oogenesis
Robert Briggs	The developmental genetics of the "o" mutation in the *Axolotl*
Burke Judd	The organization of the *Drosophila* chromosome
Joseph Ilan	Regulation of mRNA translation during development
David Epel	Molecular mechanisms of fertilization and egg activation
Walter Gehring	Genetic studies with reference to oocyte development
Peter Wellauer	Structure of ribosomal RNA

PHYSIOLOGY

I. Consultants

Arthur H. Burr, Assistant Professor, Simon Fraser University
Leon Weiss, Professor of Anatomy, Johns Hopkins University School of Medicine

II. Instructors

John J. Cebra, Professor of Biology, Johns Hopkins University
Pien-chien Huang, Associate Professor of Biochemistry, Johns Hopkins University School of Hygiene and Public Health
Ru-chih C. Huang, Associate Professor of Biology, Johns Hopkins University
Istvan Krisko, Associate Professor of Medicine, Baylor University School of Medicine
Brian J. McCarthy, Professor of Biochemistry, University of California, San Francisco
Thomas D. Pollard, Assistant Professor of Anatomy, Harvard University School of Medicine
Robert A. Prendergast, Associate Professor of Ophthalmology and Pathology, Johns Hopkins University School of Medicine

Gerald Weissmann, Professor of Medicine, New York University School of Medicine

David A. Yphantis, Professor of Biochemistry and Biophysics, University of Connecticut, Storrs

III. Special Lecturers

Frank Austin, Professor of Medicine, Harvard University School of Medicine

Ludwig Brand, Professor of Biology, Johns Hopkins University

Christopher Cordle, Department of Biology, Johns Hopkins University

Robert A. Good, Director, Sloan-Kettering Institute for Cancer Research

Daniel Goodenough, Assistant Professor of Anatomy, Harvard University School of Medicine

Elvin A. Kabat, Professor of Microbiology, Human Genetics and Development, Columbia University, College of Physicians and Surgeons

Manfred Karnovsky, Professor of Biological Chemistry, Harvard University School of Medicine

Neal Langerman, Assistant Professor of Biochemistry and Pharmacology, Tufts University School of Medicine

Fritz Lipmann, Professor of Biochemistry, Rockefeller University

John Marchalonis, Head, Laboratory of Molecular Immunology, Walter and Eliza Hall Institute for Medical Research

Peter Schiller, Laboratory of Chemical Biology, National Institutes of Health

Raymond E. Stephens, Brandeis University

Douglas Taylor, Department of Biological Sciences, State University of New York at Albany

George Weber, Professor of Pharmacology, Indiana University School of Medicine

IV. Staff Associates

Dennis Barrett, Lecturer in Zoology, University of California, Davis

Gerald A. Cole, Associate Professor of Epidemiology, Johns Hopkins University School of Hygiene and Public Health

Sylvia Hoffstein, Research Associate, New York University School of Medicine

Sarah Hitchcock, Research Associate in Biology, Brandeis University

DeLill McCarthy, Research Associate in Biochemistry, University of California Medical Center, San Francisco

Dennis Powers, Assistant Professor of Biology, Johns Hopkins University

Robley Williams, Jr., Associate Professor of Biology, Yale University

V. Course Assistants

Martha B. Barrett, University of California, Davis

Calvina Baumgartner, Johns Hopkins University

VI. Lectures

Thomas D. Pollard	I. Introduction to motile systems II. The molecular basis of muscle contraction III. Cytoplasmic actin and myosin: the molecular basis of cell motility
Sarah Hitchcock	IV. The regulation of muscle contraction and cell motility
Thomas D. Pollard	V. The relation of cytoplasmic contractile proteins to movement

	VI. Unique motile systems: bacterial flagella and peritrich spasmonemes
RAYMOND E. STEPHENS	VII. Microtubule related movements: cilia and flagella
	VIII. Microtubule related movement in the cytoplasm
DAVID A. YPHANTIS	Physical biochemistry I.
	Physical biochemistry II.
	Physical biochemistry III.
R. WILLIAMS	Oxygen-binding proteins I.
	Oxygen-binding proteins II.
LUDWIG BRAND	Application of fluorescence measurements to problems in biology
	Nanosecond fluorometry
PETER SCHILLER	Resonant energy transfer
CHRISTOPHER CORDLE	Fluorescence polarization and time dependent anistrophy
DENNIS POWERS	Physiological and genetic mechanisms of molecular adaptation to a changing environment I.
	Physiological and genetic mechanisms of molecular adaptation to a changing environment II.
DENNIS BARRETT	Control of gene expression in early development
ISTVAN KRISKO	Mechanism of peptide elongation in protein biosynthesis
	Peptide chain initiation and termination
	Mechanism of peptide bond formation—function of ribosomal proteins; species specificity
R. C. HUANG	Studies on transcription and translation of specific genes I.
	Studies on transcription and translation of specific genes II.
	Studies on transcription and translation of specific genes III.
P. C. HUANG	Fractionation and sequence analysis of radioactive nucleic acid I.
	Fractionation and sequence analysis of radioactive nucleic acid II.
	Fractionation and sequence analysis of radioactive nucleic acid III.
BRIAN MCCARTHY	Structure of interphase chromosomes
	Fractionation of chromatin—background and approaches
	Properties of fractionated chromatin
	Specificity of DNA/RNA hybridization reactions
	Unique sequence DNA/RNA hybridization in eukaryotes
ROBERT PRENDERGAST	The immune response
JOHN CEBRA	The activities of antibodies including their specific interactions with antigens
	Antibodies: the structural bases of antigen-binding specificity and their genetic control
ROBERT PRENDERGAST	Cell mediated immune responses
JOHN MARCHALONIS	Phylogeny of immune response
JOHN CEBRA	Antibody forming cells and their precursors
JOHN MARCHALONIS	Receptors on lymphocytes
ROBERT PRENDERGAST	Immunopathology
GERALD COLE	Viral infections and consequences of an immune response
GERALD WEISSMANN	Metchnikoff revisited—lysosomes and inflammation
MANFRED KARNOVSKY	Biochemical correlates of phagocytosis
GERALD WEISSMANN	Membranes—their integrity and disruption in tissue injury
	Lysosomes, microtubules and cyclic nucleotides

FRANK AUSTEN	Molecular basis of inflammation
ROBERT GOOD	Biological amplification systems in immunology
FRITZ LIPMANN	Nonribosomal polypeptide synthesis of some antibiotics
NEAL LANGERMAN	Calorimetric studies of thymidylate synthesis
DOUGLAS L. TAYLOR	The contractile basis of amoeboid movement: studies on isolated cytoplasmic models
DANIEL GOODENOUGH	Cell junctions and intracellular communication: biochemical and structural studies on isolated junctions
ELVIN KABAT	Attempts to make models of the polypeptide backbone of immunoglobulins and other proteins

EXPERIMENTAL MARINE BOTANY

I. CONSULTANTS

MARTIN GIBBS, Brandeis University
FRANK A. LOEWUS, State University of New York at Buffalo
RALPH S. QUATRANO, Oregon State University
JEROME A. SCHIFF, Brandeis University
W. RANDOLPH TAYLOR, University of Michigan
MICHAEL J. WYNNE, University of Texas

II. INSTRUCTOR

JOHN A. WEST, University of California, Berkeley

III. SPECIAL LECTURERS

JOE RAMUS, Yale University
GORDON F. LEEDALE, University of Leeds
JOHN LEE, City University of New York
G. F. PAPENFUSS, University of California, Berkeley

IV. COURSE ASSISTANTS

RICHARD WETHERBEE, University of Michigan
KENNETH MALINOWSKI, Yale University

EXPERIMENTAL INVERTEBRATE ZOOLOGY

I. CONSULTANTS

F. A. BROWN, JR., Professor of Zoology, Northwestern University
C. LADD PROSSER, Professor of Physiology, University of Illinois
CLARK P. READ, Professor of Biology, Rice University
ALFRED C. REDFIELD, Woods Hole Oceanographic Institution
W. D. RUSSELL-HUNTER, Professor of Zoology, Syracuse University
JAMES CASE, Professor of Biology, University of California, Santa Barbara

II. INSTRUCTORS

ROBERT K. JOSEPHSON, University of California, Irvine, Director of course
JAMES MORIN, University of California at Los Angeles, Associate Director of course
M. J. GREENBERG, Florida State University

JOSEPH B. JENNINGS, University of Leeds
RALPH G. JOHNSON, University of Chicago
ANN E. KAMMER, Kansas State University, Manhattan
CHARLOTTE P. MANGUM, College of William and Mary
S. K. PIERCE, University of Maryland
THOMAS J. M. SCHOPF, University of Chicago

III. SPECIAL LECTURERS

BARRY W. ACHE, Florida Atlantic University
FRANK A. BROWN, Northwestern University
FRED GRASSLE, Woods Hole Oceanographic Institution
BERND HEINRICH, University of California, Berkeley
P. HOCHACHKA, University of British Columbia
L. KIRSCHNER, Washington State University
HOWARD LENHOFF, University of California, Irvine
C. LADD PROSSER, University of Illinois
RUDI STRICKLER, Johns Hopkins University
STEVEN VOGEL, Duke University
L. WILKENS, Bryn Mawr

IV. ASSOCIATE STAFF

T. GOLDSMITH, Yale University
E. KRAVITZ, Harvard
FRED LANG, Boston University

V. COURSE ASSISTANTS

JOHN C. CORNELL, University of California, Berkeley, teaching assistant
MARY L. CORNELL, course secretary
BRIAN D. KELLER, Johns Hopkins University, teaching assistant

VI. LECTURES

R. JOSEPHSON	Introduction to Woods Hole and the course
S. PIERCE	Some thoughts on the immortal sponges
R. JOSEPHSON	Relationships between animal phyla Coelenterates
T. SCHOPF	The biology of the bryozoa
M. GREENBERG	Mollusca I. Mollusca II.
J. MORIN	Protochordates
C. MANGUM	Annelida
A. KAMMER	Arthropod I. Arthropod II.
J. MORIN	The enigmatic echinoderms
T. SCHOPF	Ecological genetics of marine invertebrates
C. MANGUM	Respiration I: gas exchange (homage to August Krogh) Respiration II: oxygen transport (from monomer to the mudflat)
S. VOGEL	Fluid and animals I: boundary layers and how they grow Fluid and animals II: flow adaptations of sessile animals
M. GREENBERG	Patterns of circulation in invertebrates
R. JOSEPHSON	The origin of nervous systems

A. Kammer	The generation of rhythmic motor outputs
J. Morin	Bioluminescence as diverse effectors
F. Lang	Arthropod neuromuscular systems
M. Greenberg	Comparative physiology and pharmacology of molluscan muscle
A. Kammer	Adaptations to environmental temperature
B. Heinrich	Thermoregulation in sphinx moths
J. Jennings	Digestive physiology in invertebrates, especially the acoelomates I.
J. Morin	Organization and coordination in colonial animals
J. Jennings	Digestive physiology in invertebrates, especially the acoelomates II.
T. Schopf	Extinction and sea floor spreading: crises in the history of life
R. Johnson	The amazing fossils of Mazon Creek or experimental invertebrate zoology 260 million years ago
S. Pierce	Salinity tolerance, osmosis and cell volume regulation
	Water balance continued, and a look at excretory systems
L. Kirschner	Ionic regulation and active transport

MARINE ECOLOGY

I. Consultants

Harlyn O. Halverson, Professor of Microbiology, Brandeis University
J. Woodland Hastings, Professor of Biology, Harvard University
Howard L. Sanders, Senior Scientist, Woods Hole Oceanographic Institution
Lawrence B. Slobodkin, Professor of Biology, State University of New York at Stony Brook
Roger Y. Stanier, Professor of Microbiology, Institut Pasteur, Paris

II. Instructors

Holger W. Jannasch, Senior Scientist, Woods Hole Oceanographic Institution, in charge of program
Jane Gibson, Associate Professor of Microbiology, Cornell University
Edward R. Leadbetter, Professor of Microbiology, Amherst College
Kenneth H. Nealson, Assistant Professor of Marine Biology, Scripps Institution of Oceanography
Ralph S. Wolfe, Professor of Microbiology, University of Illinois

III. Research Associates

Anatol Eberhard, Assistant Professor of Chemistry, Ithaca College
Carolyn Eberhard, Research Associate, Cornell University
Charles C. Remsen, Associate Scientist, Woods Hole Oceanographic Institution
Donald M. Spoon, Assistant Professor, Georgetown University
Craig D. Taylor, Postdoctoral Investigator, Woods Hole Oceanographic Institution
Edward O. Wilson, Professor of Zoology, Harvard University

IV. Laboratory Assistants

Christopher Van Raalte, Marine Biological Laboratory
Charles B. Markowitz, Amherst College

V. Special Lecturers

Richard W. Castenholz, Professor of Botany, University of Oregon
David P. Chynoweth, Associate Professor, University of Michigan
Joel C. Goldman, Assistant Scientist, Woods Hole Oceanographic Institution
Alex Keynan, Professor of Microbiology, The Hebrew University, Jerusalem
Ralph Mitchell, Professor of Applied Microbiology, Harvard University
John R. Postgate, Professor of Microbiology, The University of Sussex
John H. Ryther, Senior Scientist, Woods Hole Oceanographic Institution
Howard L. Sanders, Senior Scientist, Woods Hole Oceanographic Institution
Werner Stumm, Professor of Chemistry, Federal University, Zurich
John M. Teal, Senior Scientist, Woods Hole Oceanographic Institution

VI. Lectures

Holger W. Jannasch	Introduction to microbial ecology I
	Introduction to microbial ecology II
	Continuous culture at low substrate concentrations
	Competition in continuous culture
	Ecological aspects of denitrification
	Experiments in deep-sea microbiology
Jane Gibson	Photosynthetic microorganisms I
	Photosynthetic microorganisms II
	Movement in photosynthetic bacteria
	Biochemistry of CO_2-fixation
	Biology of *Bdellovibrio*
Edward R. Leadbetter	Making a living aerobically
	Enrichment cultures
	Aspects of microbial geochemistry I
	Aspects of microbial geochemistry II
	Microbial attack on hydrocarbons
	Amine utilization by bacteria
Kenneth H. Nealson	Regulation of bacterial bioluminescence
	Basic bacterial genetics I
	Basic bacterial genetics II
	Survey of bioluminescent systems
	Marine bacterial viruses
Ralph S. Wolfe	Anaerobic ways to make a living I
	Anaerobic ways to make a living II
	Anaerobic metabolic food chains I
	Anaerobic metabolic food chains II
	Iron bacteria
Richard W. Castenholz	Hot springs and their microorganisms I
	Hot springs and their microorganisms II
David P. Chynoweth	Sulfide formation in sediments
Joel C. Goldman	Continuous culture of photosynthetic organisms
Alex Keynan	The bacterial endospore: an example of a dormant stage in microorganisms
Ralph Mitchell	Microbial predation and chemotaxis
John R. Postgate	Ecological consequences of recent knowledge of the biochemistry of nitrogen fixation
	Survival of microorganisms
Charles C. Remsen	Fine structure survey of autotrophic and phototrophic bacteria

John H. Ryther	Aquaculture
Howard L. Sanders	Oil spill ecology
Donald M. Spoon	Protozoan ecology
Werner Stumm	Biological mediation of redox processes in seawater
	Role of redox potential and ecological milieu of microorganisms
Craig D. Taylor	Structure and methylation of co-enzyme M
John M. Teal	Experimental marsh ecology
Edward O. Wilson	Geographical ecology I
	Geographical ecology II

NEUROBIOLOGY

I. Instructors

Michael V. L. Bennett, Professor of Anatomy, Albert Einstein College of Medicine, co-director of course
John E. Dowling, Professor of Biology, Harvard University, co-director of course
Anthony L. F. Gorman, Professor of Physics, Boston University School of Medicine
Rodolfo R. Llinas, Professor of Physiology and Biophysics, University of Iowa
George Pappas, Professor of Anatomy, Albert Einstein College of Medicine
Victor P. Whittaker, Sir William Dunn Reader in Biochemistry, Cambridge University

II. Special Lecturers

George Katz, Columbia University
Malcolm Brodwick, Duke University
J. S. McReynolds, National Institute of Neurological Diseases and Stroke
F. A. Dodge, Jr., Rockefeller University
J. E. Heuser, University College, London, England
S. D. Erulker, University of Pennsylvania
F. F. Weight, National Institute of Mental Health
C. Nicholson, University of Iowa
H. Gainer, National Institutes of Health
M. J. Dowdall, Cambridge University, England
E. J. Simon, New York University Medical Center
F. Hoskin, Illinois Institute of Technology
M. Johnson, Medical Research Council, England
E. A. Kravitz, Harvard Medical School
N. W. Daw, Washington University

III. Lectures

John Dowling	Anatomical analysis of the vertebrate retina
R. Llinas	Neurocytology of the cerebellar cortex: a model of general CNS morphology
M. V. L. Bennett	The central dogma in neurobiology
George Katz	Introduction to electrophysiological measurements
A. L. F. Gorman	Introduction to anatomy and physiology of molluscan neurons
George D. Pappas	Membranes and fine structure of neurons and the fine structure of membranes
	The fine structure of the chemically-transmitting synapses

	The fine structure of electrically-transmitting synapses
S. G. Waxman	The structural specializations of the axon in relation to function
John E. Dowling	Synaptic organization of the vertebrate retina
A. M. Brown	Ionic movements in a molluscan neuron
A. L. F. Gorman	Passive membrane properties of molluscan neurons and extracellular diffusion
	Electrogenic Na^+ pump
Malcolm Brodwick	Slow potassium conductance
J. S. McReynolds	Comparative physiology of hyperpolarizing receptor potentials
R. Llinas	General properties of chemically mediated synaptic transmission—the giant synapse
F. A. Dodge, Jr.	The quantum of synaptic transmission
J. E. Heuser	Origin and fate of synaptic vesicles
S. D. Erulkar	Synaptic delay
M. V. L. Bennett	Postsynaptic actions
R. Llinas	Inhibition
M. V. L. Bennett	Receptor synapses
F. F. Weight	Synaptic potentials in sympathetic ganglia
M. V. L. Bennett	Electrical synapses
	Interpretation of spikes in the CNS
R. Llinas	Neuronal circuits in the cerebellar cortex
	Cerebellum in the regulation of movement
C. Nicholson	Field potential analysis in the cerebellum
M. V. L. Bennett	Physiology of electrical synapses
	Control of very simple effector systems
	Control of less simple effector systems
H. Gainer	Effects of synaptic activity on macromolecular metabolism of neurons
V. P. Whittaker	Biochemistry of the neurone—introduction
	Subcellular fractionation techniques applied to the nervous system
M. J. Dowdall	Synthesis and storage of neurotransmitters: parallels and contrasts
V. P. Whittaker	Dynamics of vesicle formation and discharge
E. J. Simon	Biochemical studies on the opiate and other receptors
F. Hoskin	Cholinergic drugs
H. Pollard	The structure of chromatin granules (EM and X-ray)
D. Soifer	Cyclic nucleotides in neuronal function
	Neuronal microtubules
M. Johnson	Axonal degeneration
E. A. Kravitz	Studies of synaptic chemistry in the lobster nervous system
J. E. Dowling	Review: anatomy, chemistry, and physiology of photoreceptors
	The *Limulus* lateral eye
R. L. Chappell	The median ocellus of the dragonfly: a "simple" retina
J. E. Dowling	The processing of visual information
N. W. Daw	Neurophysiology of color vision
J. E. Dowling	Gross retinal potentials, glial potentials, and the problem of visual adaptation
M. H. Goldstein	Processing of information in the auditory cortex

THE LABORATORY STAFF

Homer P. Smith, *General Manager*

Jane Fessenden, Librarian
John J. Valois, Manager, Supply Department
Lewis M. Lawday, Assistant Manager, Supply Department
Frank A. Wildes, Controller
Robert Gunning, Superintendent, Buildings and Grounds
Jim A. Hancock, Manager, Department of Research Service
Sandra E. Belanger, Assistant Editor, The Biological Bulletin

GENERAL OFFICE

Edward J. Bender
Christina Bowden
Florence S. Butz
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Joyce C. Glass
Elaine C. Perry
Lorraine A. Ruddick
Mary R. Tavares

LIBRARY

David J. Fitzgerald
Elizabeth A. Fuseler
Lenora Joseph
Holly Karalekas
Anthony J. Mahler
Theresa K. McKee

MAINTENANCE OF BUILDINGS AND GROUNDS

John F. Ackerson
Eldon P. Allen
Lee E. Bourgoin
Bernard F. Cavanaugh
James S. Clarke
John V. Day
Manuel P. Dutra
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Churney, Leon, Professor of Physiology, Louisiana State University Medical Center
Clement, Anthony C., Professor of Biology, Emory University
Clifford, Sister Adele, Professor of Biology, College of Mount St. Joseph on the Ohio
Cohen, M. W., Assistant Professor, McGill University
Cohen, Seymour S., American Cancer Society, Professor of Microbiology, University of Colorado Medical Center
Collier, J. R., Professor of Biology, Brooklyn College
Colwin, Arthur L., Professor, Queens College, City University of New York
Colwin, Laura Hunter, Professor, Queens College, City University of New York
Copeland, Donald Eugene, Professor, Tulane University
DeLanney, Louis E., Professor of Biology and Chairman, Ithaca College
Dixon, Keith, Senior Lecturer in Biology, Flinders University of South Australia
Eder, Howard A., Professor of Medicine, Albert Einstein College of Medicine
Eisen, Herman N., Professor and Head, Department of Microbiology, Washington University School of Medicine
Feingold, David S., Associate Professor of Medicine, Harvard Medical School-Beth Israel Hospital
Gabriel, Mordecai L., Dean, School of Science, Brooklyn College
German, James L., Senior Investigator, and Director, Laboratory of Human Genetics, The New York Blood Center, and Cornell University Medical College
Ginsberg, Harold S., Professor and Chairman, Department of Microbiology, University of Pennsylvania
Grant, Philip, Professor of Biology, University of Oregon
Green, James W., Professor of Physiology, Rutgers University
Gupta, Brij L., Assistant Director of Research in Zoology, Cambridge University, England
Holz, George G., Jr., Professor and Chairman, Department of Microbiology, Upstate Medical Center
Hunter, Robert Douglas, Assistant Professor, Oakland University
Ilan, Joseph, Associate Professor, Case Western Reserve University School of Medicine
Isselbacher, Kurt J., Mallinckrodt Professor of Medicine, Harvard Medical School and Chief, Gastronintestinal Unit, Massachusetts General Hospital
Karush, Fred, Professor of Microbiology, University of Pennsylvania School of Medicine
Kempton, Rudolf T., Professor Emeritus of Biology, Vassar College
Keynan, Alex, Vice President of the University, Hebrew University of Jerusalem
Kirschenbaum, Donald M., Associate Professor, College of Medicine, Downstate Medical Center
Kosower, Edward M., Professor of Chemistry, Tel-Aviv University
Kosower, Nechama S., Associate Professor, Tel-Aviv University
Kuffler, Stephen W., Robert Winthrop Professor of Neurobiology and Department Chairman, Harvard Medical School
Laderman, Aimlee D., Instructor, Ramapo College of New Jersey
Landsberger, Frank R., Assistant Professor of Chemistry, Indiana University
Laufer, Hans, Professor of Biology, University of Connecticut
LeFevre, Paul G., Professor of Physiology and Biophysics, State University of New York at Stony Brook
Leighton, Joseph, Professor and Chairman, Department of Pathology, The Medical College of Pennsylvania
Lenard, John, Associate Professor, Rutgers Medical School
Lochhead, John H., Professor of Zoology, University of Vermont
Marsland, Douglas A., Research Professor Emeritus, New York University
Meins, Frederick, Jr., Assistant Professor of Biology, Princeton University
Monroy, Alberto, Professor and Director, C.N.R. Laboratory of Molecular Embryology, Italy

Morrell, Frank, Professor of Neurology, Rush Medical College
Odell, Garrett M., Assistant Professor of Mathematics, Rensselaer Polytechnic Institute
Oschman, James L., Assistant Professor, Northwestern University
Palmer, John D., Chairman and Professor of Biology, New York University
Plocke, Donald J., Associate Professor and Chairman, Boston College
Rosenkranz, Herbert S., Professor of Microbiology, Columbia University
Roth, Jay S., Professor of Biochemistry, University of Connecticut
Rowland, Lewis P., Professor and Chairman of Neurology, University of Pennsylvania
Rubinow, Sol I., Professor of Biomathematics, Cornell University Medical College
Saunders, John W., Jr., Professor of Biological Sciences, State University of New York at Albany
Schlesinger, R. Walter, Professor and Chairman, Department of Microbiology, Rutgers Medical School, College of Medicine and Dentistry of New Jersey
Scott, Allan C., Professor, Colby College
Seecof, Robert, Head, Department of Developmental Biology, City of Hope National Medical Center
Shedlovsky, Theodore, Professor Emeritus, Rockefeller University
Shemin, David, Professor of Biochemistry, Northwestern University
Sherman, Irwin W., Professor of Zoology, University of California, Riverside
Sieburth, John McN, Professor of Microbiology and Oceanography, University of Rhode Island
Sonnenblick, B. P., Professor of Zoology, Rutgers University
Steinberg, Malcolm S., Professor, Princeton University
Strittmatter, Philipp, Professor and Head, Department of Biochemistry, University of Connecticut Health Center
Tanzer, Marvin L., Associate Professor of Biochemistry, University of Connecticut Medical School
Terebey, Nicholas, Assistant Professor of Anatomy, New York University
Tucker, Gail Susan, Postdoctoral, Columbia University College of Physicians and Surgeons
Twarog, Betty M., Professor, Tufts University
Tweedell, Kenyon S., Professor of Biology, University of Notre Dame
Wainio, Walter, Professor of Biochemistry, Rutgers—The State University of New Jersey
Waksman, Byron H., Professor of Microbiology, Yale University
Wall, Patrick D., Professor of Anatomy, University College, London
Webb, H. Marguerite, Professor and Chairman, Department of Biological Sciences, Goucher College
Weiss, Leon, Professor of Anatomy, Johns Hopkins Medical School
Wheeler, George E., Professor of Biology, Brooklyn College
Wichterman, Ralph, Professor of Biology, Temple University
Wiercinski, Floyd J., Professor of Biology, Northeastern Illinois University
Wilson, Thomas Hastings, Professor of Physiology, Harvard Medical School
Wittenberg, Jonathan B., Professor of Physiology, Albert Einstein College of Medicine
Wolken, Jerome J., University Professor of Biophysics, Carnegie-Mellon University
Yntema, Chester L., Professor of Anatomy, State University of New York, Upstate Medical Center

Students, 1973

All students listed completed the formal course program. Asterisk indicates completing post-course research program.

ECOLOGY

Balch, William E., University of Illinois
*Becvar, James E., Harvard University
*Brewer, Linda A., Stanford University
Curtis, Paul R., Eisenhower College
Fox, George E., Syracuse University
*Greaney, George S., Johns Hopkins University
*Greenberg, Everett P., University of Massachusetts

Hyde, Camille L., University of Michigan
*Keenan, Katherine, City College of New York
*Kiefer, Lynda A., University of West Florida
McKay, Melvin G., Dalhousie University
Schwalbe, Cecil R., University of Arizona
*Stein, Carol L., Harvard University
*Stoll, Denise R., Boston University
Walton, Diana S., University of Illinois

EMBRYOLOGY

*Baker, J. B., University of Hawaii
*Beckendorf, S. K., California Institute of Technology
*Chyba, D. E., University of Wisconsin
*Cottrell, S. F., Rutgers University
*Feldherr, C., University of Pennsylvania
*Gabrielli, F., University of Wisconsin
*Galau, G. A., California Institute of Technology
*Hall, L., University of British Columbia
*Hobart, P. M., Wesleyan University
*Hunsley, J. R., Michigan State University
*Kahn, E. B., Northwestern University
*Loomis, M. R., Indiana University
*Newman, S. A., University of Chicago
*Norman, J. A., University of California
*Ostrer, H., Columbia University
*Pinon, R., University of Washington
*Snyder, B. W., University of Michigan
*Steel, L. F., Cornell University
*Wyman, A. R., Harvard Medical School
*Zuniga, M., Yale University

EXPERIMENTAL BOTANY

Agnes, Sister Cecilia, Regis College
Averback, Rae Rachael, Bard College
Brawley, Susan Howard, Wellesley College
Dean, John M., University of Massachusetts
Dehn, Paula Faye, DePauw University
Fulton, Allice Bordwell, Brown University
Levandoski, Dennis, Brooklyn College
Lunn, Patricia Ann, Drew University
Postelle, Rebekah Ann, Tennessee Wesleyan College
Riley, William Denis, University of Massachusetts, Boston
Scott, Gordon, University of Massachusetts
Stead, Richard B., University of Wisconsin
Straus, John William, Earlham College
Williams, Deborah W., Harvard University, Radcliffe College
Yen, Duenying, Jennette, Bryn Mawr College

PHYSIOLOGY

*Bailey, Stephen, Washington University
Barnitz, Joy, New College
Bubbers, J. Eric, Johns Hopkins University
*Burns, Roy G., University of Edinburgh
Cohen, Robert M., Amherst College
Davis, Francis M., Yale University

*Dean, Paula, Johns Hopkins University
DeMoss, Harvard Medical School
Dodge, Robert H., Texas A & M University
*Fallon, Ann M., Yale University
*Findley, Robert, Yale University
Fitzgerald, Gregory J., University of Dayton
Fujiwara, Keigi, University of Pennsylvania
*Grainger, James L., University of California, Santa Cruz
Hackett, Charles J., Wayne State University
Haskell, Amy, College of William and Mary
Hoff, Steven, F., University of California, Los Angeles
Hogg, James C., McGill University
*Hoppensteadt, Frank, New York University
*Hunt, Richard K., University of Pennsylvania
Johmann, Carol A., University of Rochester
Kam, Zvi, University of California, San Diego
Kirby, Christine A., University of Pittsburgh
Kleyn, John G., University of Puget Sound
Kuppers, Rudolf C., University of California, Northridge
Litchfield, William J., University of Illinois
*Lovgren, Timo N-E., University of New Mexico
Madison, Carolyn R., Newark State College
*McCumber, Larry Joe, University of Florida
Mednieks, Maija I., DePaul University
Miller, Stella C., Johns Hopkins University
*Parker, Wendy L., City College of New York
Reimer, Ernest M., Simon Fraser University
Ribolini, Ann, State University of New York at Stony Brook
*Rice, Charles M., University of California, Davis
*Rothblum, Lawrence I., Hahnemann Medical College
Rutz, Richard E., Jr., Michigan State University
*Sanger, Joseph W., University of Pennsylvania
*Schacher, Samuel M., Columbia University
*Tuan, Rocky S-c., Rockefeller University
*Weiss, Martin B., University of Massachusetts, Amherst

INVERTEBRATE ZOOLOGY

Bartberger, Carol A., University of Maryland
Bollen, Andrew W., Harvard College
*Bouton, Rebecca K., Florida Atlantic University
Burstein, Neal, Stanford University
*Collins, James, Marquette University
Crabtree, Robert L., Ohio University
Day, Deborah, Universite' Aix-en-Provence, Marseille
Donohue, Neil, Drew University
Dwyer, Ellen, Wilson College
*Epstein, Kerry, University of Oregon
*Fowler, Velia, Oberlin College
Freadman, Marvin, College of William and Mary
Friedlander, Michael, University of Illinois
*Girsch, Steve, Harvard University
Hall, Kathleen, University of Minnesota
Herbst, Gary N., University of Wisconsin
Jenkins, Carole, University of Pittsburgh
Kemp, William M., University of Florida
Leonard, Stephanie, Oakland University

Ling, Tsin Yuen Jeanne, University of Maryland
*Martini, Mary, University of California, Santa Barbara
*Mykles, Donald L., University of California, Berkeley
Nathn, Sharon, Simmons College
Osbon, Maria, California State University, San Francisco
Price, Christopher H., Syracuse University
Raderman, Randie, State University of New York
*Reingold, Stephen C., Cornell University
*Stolper, David, Upstate Medical Center
*Swenson, Randolph P., Washington State University
Wheeler, Patricia, University of California, Irvine
Zemke, Stephen, Wesleyan University

NEUROBIOLOGY

Bohr, Vilheim Alfred, University of Copenhagen
Caldwell, John Henderson, Washington University
Counter, S. Allen, Harvard University
Eaton, Robert Charles, University of California
Feldman, Jack L., University of Chicago
Forsythe, David William, Princeton University
Heyer, Carolyn Baker, University of Iowa
Kimble, Judith E., University of Copenhagen
Kovac, Marc P., Stanford University
Pollard, Harvey B., National Institutes of Health
Samples, John Randall, Stanford University
Srinivasan, Mandyam Veerambudi, Yale University

FRONTIERS IN RESEARCH AND TEACHING PROGRAM

Clark, Vernon, North Carolina Central University
Stephens, Lee B., California State University

4. Fellowships and Scholarships, 1973

Bio Club Scholarship:

Katherine Keenan, Ecology Course
Wendy L. Parker, Physiology Course

Gary H. Calkins Scholarship:

Rebecca K. Bouton, Invertebrate Zoology Course
Carole Jenkins, Invertebrate Zoology Course
Michael Friedlander, Invertebrate Zoology Course
Randie Raderman, Invertebrate Zoology Course
Patricia Wheeler, Invertebrate Zoology Course

5. Training Program

Fertilization and Gamete Physiology Research Training Program

I. Instructors

Charles B. Metz, University of Miami, Program Director
Bjorn Afzelius, University of Stockholm, Sweden
Tom Humphreys, University of Hawaii
Joe Rosenbaum, Yale University
William Telfer, University of Pennsylvania
Robin Wallace, Oak Ridge National Laboratory

II. Assistants

Diane Robins, EM Assistant
Sylvia Ann Thompson Rauback, Program Secretary
Robert Spindell, Photographic Assistant

III. Trainees

Lester I. Binder, Yale University
Marguerite F. Knebel Bohm, University of Kansas
Donald C. Chang, Rice University
Christopher T. Cordle, Johns Hopkins University
Glenn E. Daniels, University of Pennsylvania
Mark N. Hill, Miami University
Paul A. Lefebvre, Rockefeller University
Anthony L. Mescher, Ohio State
Frederick W. Miller, Miami University
Phoebe Mounts, University of Pittsburgh
Paul A. Rosenberg, Albert Einstein Medical School
Barry W. Simon, Tulane University
Roger D. Sloboda, Rensselaer Polytechnic Institute
Andrea J. Tenner, University of California, San Diego
Donna L. Vogel, Albert Einstein Medical School
George B. Witman III., University of Chicago

IV. Lectures

Charles Metz	Mammalian sperm antigens and their roles in reproduction
Marjorie Crandall	Molecular complementary of yeast glycoprotein mating factors
Bjorn Afzelius	Sperm morphology and fertilization biology
Herbert Schuel	Molecular constituents of egg cortical granules. Function of tryptic proteinase in fertilization
Joel Rosenbaum	Flagellar regeneration in *Chlamydomonas*: A system for studying the *in vivo* synthesis and assembly of microtubule
George Witman	Directionality of microtubule assembly during flagellar regeneration in *Chlamydomonas*
Lester Binder	Directionality of microtubule assembly *in vitro*
Tom Humphreys	Ribosomal and DNA-like RNA metabolism in sea urchin embryos
Sheldon J. Segal	Specific and heterospecific transfer of hormone action by RNA
Mareo Burgos	Maturation of the mammalian spermatozoon

Excitable Membrane Physiology and Biophysics Training Program

I. Consultants

W. J. Adelman, Jr., National Institute of Neurological Diseases and Stroke
K. S. Cole, National Institute of Neurological Diseases and Stroke
J. W. Moore, Duke University Medical Center
L. J. Mullins, University of Maryland, School of Medicine

II. Instructors

D. E. Goldman, Program Director, Medical College of Pennsylvania
F. A. Dodge, Associate Director, IBM Corp.
K. S. Cole, National Institute of Neurological Diseases and Stroke
J. W. Moore, Duke University Medical Center
D. Gilbert, National Institute of Neurological Diseases and Stroke
L. J. Mullins, University of Maryland, School of Medicine
M. P. Blaustein, Washington University

W. K. Chandler, Yale University School of Medicine
W. J. Adelman, Jr., National Institute of Neurological Diseases and Stroke
T. Narahashi, Duke University Medical Center
E. S. Masoro, Medical College of Pennsylvania
M. M. Dewey, State University of New York at Stony Brook
W. K. Blasie, University of Pennsylvania
H. Lecar, National Institute of Neurological Diseases and Stroke
L. E. Moore, Case Western Reserve University
C. L. Prosser, University of Illinois
C. M. Armstrong, University of Rochester
R. De Haan, Carnegie Institution of Washington
J. Tasaki, National Institute of Mental Health
L. Cohen, Yale University School of Medicine
H. Fishman, State University of New York at Albany
J. Y. Lettvin, Massachusetts Institute of Technology
R. S. Manalis, University of Cincinnati

III. Assistants

P. Greif, Haverford College
G. Stetten, Harvard College

IV. Trainees

R. F. Abercrombie, University of Maryland
D. M. Easton, Florida State University
W. F. Gilly, Washington University
R. S. Kass, University of Michigan
R. D. Nathan, University of California, Los Angeles
T. W. Pearson, University of California, Davis
J. F. Poduslo, University of Pennsylvania
J. M. Schaeffer, Purdue University
E. E. Swenberg, Brooklyn College
R. E. Yantorno, University of Pennsylvania

V. Lectures

D. E. Goldman	Physical and electrochemistry of membranes I.
	Physical and electrochemistry of membranes II.
	Physical and electrochemistry of membranes III.
K. S. Cole	Electrical properties of membranes
	The strategy of the voltage clamp
J. W. Moore	Operational amplifiers
	Preamplifiers
	Voltage clamp circuits
D. Gilbert	The biology of squid
L. J. Mullins	Passive transport
M. P. Blaustein	Active transport
F. A. Dodge	Cable theory
W. K. Chandler	Voltage clamp currents in squid
	The Hodgkin-Huxley formulation
	Univalent ions and selectivity
D. E. Goldman	Divalent cations
W. J. Adelman, Jr.	The periaxonal space
L. E. Moore	Myelinated nerve
J. W. Moore	TTX and excitable membranes
W. J. Masoro	Chemical composition of membranes
M. M. Dewey	Electron microscopy and membrane structure

H. Lecar	Artificial membranes
W. K. Blasie	X-ray diffraction of membranes
T. Narahashi	Action of membrane "stabilizers"
	Action of membrane "labilizers"
D. E. Goldman	Excitability models I.
	Excitability models II.
	Excitability models III.
C. M. Armstrong	Models for Na channels
	Models for K channels
W. K. Chandler	Striated muscle I.
	Striated muscle II.
	Striated muscle III.
C. L. Prosser	Smooth muscle
R. De Haan	Electrical activity and entrainment of heart cells in culture
D. E. Goldman	Surface charges in axon membranes
	Non-electrical stimulation of axons
I. Tasaki	Axon perfusion and membrane behavior
L. Cohen	Optical properties of axons
H. Fishman	Noise in the squid axon
J. Y. Lettvin	The relation between semi-conductors and electrolytes with respect to membranes

Research Program in Experimental Marine Botany

I. Senior Investigators

Martin Gibbs, Brandeis University
Frank A. Loewus, State University of New York at Buffalo
Ralph S. Quatrano, Oregon State University
Jerome A. Schiff, Brandeis University
Michael J. Wynne, University of Texas, Austin

II. Associate Investigators

Hans Gaffron, Florida State University
Robert C. Hodson, University of Delaware
Mary W. Loewus, State University of New York at Buffalo

III. Junior Investigators

Amitzur Ben-Amotz, Weizmann Institute of Science, Israel
Scott E. Bingham, University of Rhode Island
Janice Ann Derr, Washington University, St. Louis
Franklin Fong, University of California at Riverside
Arthur C. Ley, Scripps Institute of Oceanography, San Diego
Dwight G. Peavey, Brandeis University, Waltham, Massachusetts
Dennis Shevlin, Duke University, Durham, North Carolina
Stuart G. Siddell, University of Warwick, England
Nicole Leveque, Pasteur Institute, Paris, France
David N. Young, University of California at Berkeley

IV. Lectures

D Cheney	Current research in *Eucheuma*, a carrageenan producer from the south
N. Bishop	Vitamin E and photosynthesis
E. McCandless	Biochemical consequences of the sex life of *Chondrus*
D. A. Walker	Some aspects of photosynthetic induction
A. Trebst	Energy conservation in photosynthetic electron flow

6. Tabular View of Attendance, 1969–1973

	1969	*1970*	*1971*	*1972*	*1973*
Investigators—Total	566	532	554	561	523
Independent	310	324	322	328	312
Library Reader	68	73	76	76	86
Research Assistants	188	135	156	157	125
Students—Total	118	142	130	119	123
Invertebrate Zoology	35	41	29	38	32
Embryology	20	28	28	19	20
Physiology	30	31	33	31	41
Experimental Botany	16	19	22	14	15
Ecology	17	23	18	17	15
Trainees—Total	29	33	44	46	50
Total Attendance	713	707	728	726	696
Less Persons represented in two categories	5	0	0	1	0
	708	707	728	725	696
Institutions Represented—Total	187	191	219	210	239
Foreign Institutions Represented	24	21	27	25	40

7. Institutions Represented, 1973

Alabama, University of
Albert Einstein College of Medicine
American Museum of Natural History
Amherst College
Arizona, University of
Bard College
Beth Israel Hospital
Boston City Hospital
Boston College
Boston University
Boston University School of Medicine
Brandeis University
Brooklyn College, The City University of New York
Brown University
Bryn Mawr College
California, University of, Berkeley
California, University of, Davis
California, University of, Irvine
California, University of, Los Angeles
California, University of, Riverside
California, University of, San Diego
California, University of, San Francisco
California, University of, Santa Barbara
California, University of, Santa Cruz
California Institute of Technology
California State University
California State University, Long Beach
Carnegie Institution of Washington
Carnegie-Mellon University
Case Western Reserve University
Chicago, University of
Cincinnati, University of
Citadel, The
City College, The City University of New York
City of Hope National Medical Center
Colby College
College of Mount St. Joseph on the Ohio
College of William and Mary
Colorado, University of
Colorado, University of, Medical Center
Columbia University
Columbia University, College of Physicians and Surgeons
Connecticut, University of
Connecticut, University of, Health Center
Connecticut, University of, Medical School
Cornell University
Cornell University Medical College
Dartmouth College
Dayton, University of
Delaware, University of
Delaware State College
De Paul University
De Pauw University
Drew University
Duke University
Duke University Medical Center
Earlham College
Eisenhower College

Emory University
Florida, University of
Florida Atlantic University
Florida State University
Georgetown University
Georgia, University of
Goucher College
Gulf Coast Research Laboratory
Hahnemann Medical College
Hartwick College
Harvard Medical School
Harvard University
Haverford College
Hawaii, University of
Herbert Lehman College, The City University of New York
Houston, University of
Howard University
Hunter College, The City University of New York
Illinois, University of
Illinois Institute of Technology
Indiana University
Institute for Cancer Research, The
Institute for Muscle Research
Iowa, University of
Ithaca College
Johns Hopkins University, The
Johns Hopkins University, The, School of Hygiene
Johns Hopkins University, The, School of Medicine
Juniata College
Kansas, University of
Kansas State University
Kentucky, University of
Kenyon College
Louisiana State University
Louisiana State University Medical Center
Lowell Technological Institute
Marquette University
Marine Research Foundation, Inc.
Maryland, University of
Maryland, University of, School of Medicine
Massachusetts, University of
Massachusetts General Hospital
Massachusetts Institute of Technology
Medical College of Ohio at Toledo
Medical College of Pennsylvania
Mellon Institute of the Carnegie-Mellon University
Miami, University of
Miami, University of, School of Medicine
Michigan, University of
Michigan State University
Middlebury College
Minnesota, University of
National Bureau of Standards
National Institute of Mental Health
National Institute of Neurological Diseases and Stroke
National Institutes of Health
New College
New Hampshire, University of
New Mexico, University of
New York Blood Center, The
New York University
New York University Medical Center
New York University School of Medicine
Newark State College
North Carolina, University of, Chapel Hill
North Carolina Central University
North Carolina State University, Raleigh
Northeastern Illinois University
Northwestern University
Notre Dame, University of
Oak Ridge National Laboratory
Oakland University
Oberlin College
Ohio State University
Ohio University
Oregon, University of
Oregon State University
Pennsylvania, University of
Pennsylvania, Hospital of the University of
Pennsylvania, University of, School of Medicine
Pittsburgh, University of
Population Council, The
Portland State University
Princeton University
Puget Sound, University of
Purdue University
Queens College, The City University of New York
Radcliffe College
Ramapo College of New Jersey
Regis College
Rensselaer Polytechnic Institute
Rhode Island, University of
Rice University
Rochester, University of
Rochester, University of, Medical School
Rockefeller University, The
Rush Medical College
Rutgers -The State University
Rutgers University Medical School
Scripp's Institution of Oceanography
Simmons College
Stanford University
State University of New York, Downstate Medical Center
State University of New York, Upstate Medical Center
State University of New York at Albany
State University of New York at Binghamton

State University of New York at Buffalo
State University of New York at Stony Brook
Syracuse University
Temple University
Temple University Health Sciences Center
Temple University Medical School
Tennessee Medical Units, University of
Tennessee Wesleyan College
Texas A & M University
Texas Christian University
Texas, University of, Arlington
Texas, University of, Austin
Trinity College
Tulane University
Upsala College
Vanderbilt University
Vassar College
Veterans Administration Hospital, Brooklyn
Virginia, University of
Washington University
Washington University School of Medicine
Wayne State University
Wellesley College
Wesleyan University
West Florida, University of
Western Kentucky University
Wilson College
Wisconsin, University of
Wistar Institute
Woods Hole Oceanographic Institution
Worcester Polytechnic Institute
Yale University
Yale University School of Medicine

FOREIGN INSTITUTIONS REPRESENTED, 1973

Basel, The University of, Switzerland
British Columbia, University of, Canada
Calgary, University of, Canada
Cambridge, University of, England
CNR Laboratory of Molecular Embryology, Italy
Copenhagen, University of, Denmark
Dalhousie University, Canada
Edinburgh University Scotland
Flinders University of South Australia, Australia
Glasgow University, Scotland
Hebrew University Medical School, Israel
Jichi Medical School, Japan
Karolinska Institute, Sweden
Leeds, University of, England
London University, England
Max Planck Institute for Biophysical Chemistry, West Germany
McGill University, Canada
McMaster University, Canada
Medical Research Council, Toxicology Research Unit, England
Montreal, University of, Canada
Münster, University of, Germany
Nagoya City University, Japan
Nara Medical University, Japan
Ottawa, University of, Canada
Pasteur Institute, France
Queen Elizabeth College, England
Queen Mary College, England
Simon Fraser University, Canada
Stockholm University, Sweden
Tel-Aviv University, Israel
Tokyo Kyoiku University, Japan
Toronto, University of Canada
Universita degli Studi di Milano, Italy
Université Laval, Canada
University College, England
Walter and Eliza Hall Institute of Medical Research, Australia
Warwick, University of, England
Weizmann, Institute of Science, Israel
Wenner-Gren Institute, Sweden
York University, England

8. FRIDAY EVENING LECTURES, 1973

June 28

SAUL ROSEMAN. . . . . .Sugar transport in bacteria
The Johns Hopkins University

July 6

JOSEPH G. GALL. . . . . .Repetitive DNA in *Drosophila*
Yale University

July 13

Keith R. Porter .... Studies on cell topography by scanning microscopy
University of Colorado

July 19

Patrick D. Wall .... Somatic sensory mechanisms in relation to sensation
University College, London
Alexander Forbes Lecturer at MBL

July 20

Patrick D. Wall .... Pain mechanisms as a special example of somatosensory mechanisms

July 27

Sheldon J. Segal .... The population problem: a progress report
The Population Council

August 3

John B. Buck .... The seventh sense: rhythm and synchrony in fireflies and man
National Institutes of Health

August 10

Annemarie Weber .... Information transfer in the actin filament
University of Pennsylvania,
Medical School

August 17

Jean G. Baer .... Forging the first link
University of Neuchatel
Agassiz Memorial Lecturer

August 24

M. S. Bretscher .... How are membranes put together?
Medical Research Council,
Cambridge

9. Members of the Corporation, 1973

Including Action of 1973 Annual Meeting

Life Members

Adolph, Dr. Edward F., University of Rochester School of Medicine and Dentistry, Rochester, New York 14627

Beam, Dr. Harold W., Department of Zoology, State University of Iowa, Iowa City, Iowa 52240

Behre, Dr. Elinor M., Black Mountain, North Carolina 28711

BERTHOLF, DR. LLOYD M., 1228 Gettysburg Drive, Bloomington, Illinois 61701
BODANSKY, DR. OSCAR, Department of Biochemistry, Memorial Cancer Center, 444 East 68 Street, New York, New York 10021
BRADLEY, DR. HAROLD C., 2639 Durant Avenue, Berkeley, California 94704
BROWN, DR. DUGALD E. S., Cape Haze, Box 426, Placida, Florida 33946
BURDICK, DR. C. LALOR, The Lalor Foundation, 4400 Lancaster Pike, Wilmington, Delaware 19805
COLE, DR. ELBERT C., 2 Chipman Park, Middlebury, Vermont 05753
COWDRY, DR. E. V., 4580 Scott Avenue, St. Louis, Missouri 63110
DILLER, DR. IRENE C., 2417 Fairhill Avenue, Glenside, Pennsylvania 19038
DILLER, DR. WILLIAM F., 2417 Fairhill Avenue, Glenside, Pennsylvania 19038
FERGUSON, DR., JAMES K. W., 56 Clarkshaven St., Thornhill, Ontario, Canada
FURTH, DR. JACOB, 99 Fort Washington Ave., New York, New York 10032
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V. REPORT OF THE LIBRARIAN

The library book section has expanded so rapidly that more room had to be found in the Lillie Building for additional shelving. Over 100 books were purchased with the Associates 1971 gift and a total of 710 books were added to this section this year.

The Woods Hole Oceanographic Institution received a substantial amount of money for books and will order approximately 1,500 volumes in 1974. Therefore Room 306 will be converted to library stack space and all books relating to Ocean Engineering, Physics, Mathematics and Marine Policy will be shelved temporarily in that room on the third floor.

Interlibrary loan requests continue to come in at a greater rate than the previous year. We average about 20 to 30 requests per day.

Two staff members left for other positions—Virginia Brandenberg, on the library staff for ten years, left to work in the Boston area and Cathy Glynn, Periodicals Librarian, also resigned. They have been replaced by Elizabeth Fuseler, formerly a librarian at the University of Pennsylvania, and Terry McKee, a 1973 graduate of Skidmore College.

The library is currently receiving 2,624 serial titles and holdings now total 151,764 volumes.

VI. REPORT OF THE TREASURER

The market value of the General Endowment Fund and the Library Fund at December 31, 1973, amounted to $2,154,552 and the corresponding securities are entered in the books at a value of $1,633,192. This compares with values of $2,579,583 and $1,624,597 respectively, at the end of the preceding year. The average yield on the securities was 4.18% of the market value and 5.52% of the book value. Uninvested principal cash was $2,433. Classification of the securities held in the Endowment Fund appears in the Auditor's Summary of Investments.

The market value of the Pooled Securities at December 31, 1973, amounted to $1,088,355 as compared to book values of $930,340. These figures compare with values of $1,238,943 and $922,227 respectively, at the close of the preceding year. The average yield on the securities was 4.00% of the market value and 4.73% of the book value. Uninvested principal cash was in the amount of $823.

The proportionate interest in the Pool Fund Account of the various funds, as of December 31, 1973, is as follows:

Pension Funds	30.41%
General Laboratory Investment	28.04%
F. R. Lillie Memorial Fund	2.04%
Other:	
Bio Club Scholarship Fund	0.53%
Rev. Arsenius Boyer Scholarship Fund	0.64%
Garry N. Calkins Fund	0.67%
Allen R. Memhard Fund	0.12%
Lucretia Crocker Fund	2.21%
E. G. Conklin Fund	0.37%
Jewett Memorial Fund	0.19%
M. H. Jacobs Scholarship Fund	0.27%
Herbert W. Rand Fellowship	18.84%
Mellon Foundation	8.89%
Mary Rogick Fund	1.94%
Swope Foundation	4.90%

Donations from MBL Associates for 1973 amounted to $17,250 as compared with $10,755 for 1972. Unrestricted gifts from foundations, societies and companies amounted to $27,967.

During the year we administered the following grants and contracts:

Investigators	*Training*	*MBL Institutional*
4 NIH	3 NIH	1 NSF
5 NSF	1 Research Corp.	
1 EPA		
1 MWPC		
1 NOAA		
1 API		
1 Damon Runyon Cancer		
1 Merrill Trust		
15	4	1

Most of the federally funded research grants and contracts provided for reimbursement of indirect costs on a cost per square foot basis for the laboratory space assigned to each project. A provisional rate of $12.25 per square foot was in effect throughout the year. Training courses supported by NIH grants were funded for indirect costs at a rate of 8% of allowable direct costs.

The following is a statement of the auditors:

To the Trustees of Marine Biological Laboratory, Woods Hole, Massachusetts:

We have examined the balance sheet of Marine Biological Laboratory as of December 31, 1973 and the related statements of operating expenditures and income and funds for the year then ended. Our examination was made in accordance with generally accepted auditing standards, and accordingly included such tests of the accounting records and such other auditing procedures as we considered necessary in the circumstances. We previously examined and reported on the 1972 financial statements.

In our opinion, the aforementioned financial statements (pages 75 to 79) present fairly the financial position of Marine Biological Laboratory at December 31, 1973 and 1972, and its operating expenditures and income for the years then ended, and the changes in its funds for the year ended December 31, 1973, and in conformity with the accounting principles referred to in Note A to the financial statements applied on a consistent basis.

The supplementary schedules (page 80) included in this report were obtained from the Laboratory's records in the course of our examination and, in our opinion, are fairly stated in all material respects in relation to the financial statements taken as a whole.

Boston, Massachusetts
March 22, 1974

COOPERS & LYBRAND

It will be noted from the operating statement that the Laboratory activities for the year under review, amounted to a figure of a little over 1.8 million dollars, which amount is 150 thousand more than the preceding year.

Alexander T. Daignault,
Treasurer

MARINE BIOLOGICAL LABORATORY

Balance Sheets

December 31, 1973 and 1972

Investments	*1973*	*1972*
Investments held by Trustee:		
Securities, at cost (approximate market quotation, 1973—$2,154,552; 1972—$2,579,583)	$ 1,633,192	$ 1,624,597
Cash	2,433	1,850
	1,635,625	1,626,447
Investments of other endowment and unrestricted funds:		
Pooled investments, at cost (approximate market quotation, 1973—$1,088,355; 1972—$1,238,943)	930,340	922,227
Other investments	1,366,230	1,094,752
Cash	1,571	31,516
Due from current fund–	189,168	131,927
	$ 4,122,934	$ 3,806,869
Plant Assets		
Land, buildings, library and equipment, at cost	12,454,336	12,453,416
Less allowance for depreciation (Note A)	2,726,028	2,444,052
	$ 9,728,308	$10,009,364
Current Fund Assets		
Cash	65,310	388,130
Accounts receivable (U. S. Government, 1973—$33,739; 1972—$88,186)	252,589	235,903
Inventories of supplies and bulletins	48,533	43,514
Other assets	5,912	5,581
Due to endowment funds	(189,168)	(131,927)
	$ 183,176	$ 541,201

MARINE BIOLOGICAL LABORATORY

BALANCE SHEETS

December 31, 1973 and 1972

Invested Funds	*1973*	*1972*
Endowment funds given in trust for benefit of Marine Biological Laboratory	$ 1,635,625	$ 1,626,447
Endowment funds for awards and scholarships:		
Principal	449,777	427,702
Unexpended income	65,715	60,795
	515,492	488,497
Unrestricted funds functioning as endowment	1,726,603	1,435,918
Retirement fund	357,439	316,512
Pooled investments—accumulated loss	(112,225)	(60,505)
	$ 4,122,934	$ 3,806,869

Plant Funds		
Funds expended for plant, less retirements	12,454,336	12,453,416
Less allowance for depreciation charged thereto	2,726,028	2,444,052
	$ 9,728,308	$10,009,364

Current Fund Liabilities and Balances		
Accounts payable and accrued expenses	20,677	26,333
Advance subscriptions	35,899	28,293
Unexpended grants—research	39,987	77,774
Unexpended balances of gifts for designated purposes	124,124	87,949
Current fund	(37,511)	320,852
	$ 183,176	$ 541,201

The accompanying note is an integral part of the financial statements.

Note A—*Accounting Principles:* The following accounting principles have been reflected in the accompanying financial statements:

1. Investments are stated at cost.
2. Investment income is recorded on a cash basis.
3. Operating income is recorded when earned.
4. Expenses are recorded on an accrual basis.
5. Depreciation has been provided for plant assets at annual rates ranging from 1% to 5% of the original cost of the assets.

MARINE BIOLOGICAL LABORATORY

Statement of Operating Expenditures and Income

Years Ended December 31, 1973 and 1972

	1973						
	Salaries and Wages	*Other Costs and Expenses*	*Depreciation (Note A)*	*Total*	*Charged to Grants*	*1973 Total*	*1972*
Operating expenditures:							
Instruction		$ 39,391	$ 75,584	$ 114,975	$230,972	$ 345,947	$ 375,633
Research		48,118	80,884	129,002	332,805	461,807	436,241
Dormitories	$ 24,890	50,884	100,980	176,754		176,754	166,876
Dining		103,376		103,376		103,376	108,745
Library	47,379	21,327	17,701	86,407		86,407	74,253
Back sets, serials and binding		71,915		71,915		71,915	60,674
Biological Bulletin	6,881	54,221		61,102		61,102	55,407
Support services:							
Apparatus	56,812	75,089		131,901		131,901	120,961
Supply	89,865	76,735	4,718	171,318		171,318	154,929
Administration	113,976	137,002		250,978		250,978	197,092
Plant operation	171,998	163,910	2,108	338,016		338,016	311,710
Grant expenditures for support services					15,810	15,810	19,296
Other		42,309		42,309		42,309	38,293
	$511,801	$884,277	$281,975	1,678,053	579,587	2,257,640	2,120,110

	Fees	*Other*				
Income:						
Instruction	$106,260		106,260	230,972	337,232	359,087
Research	355,016		355,016	332,805	687,821	543,031
Dormitories		$200,052	200,052		200,052	179,321
Dining		114,058	114,058		114,058	121,083
Library	27,721	42,549	70,270		70,270	66,359
Biological Bulletin		60,511	60,511		60,511	57,139
Support services:						
Apparatus		51,028	51,028		51,028	49,724
Supply		72,090	72,090		72,090	62,973
Administration		23,844	23,844		23,844	21,554
Investment income		160,435	160,435		160,435	150,864
Gifts used for current expense		39,217	39,217		39,217	67,302
Allowance for indirect costs		40,648	40,648		40,648	39,393
Grants for support services				15,810	15,810	19,296
Other		15,529	15,529		15,529	6,937
	$488,997	$819,961	1,308,958	$579,587	1,888,545	1,744,063
Excess of current expenditures and depreciation over current income			369,095		369,095	376,047
Reduction in plant funds for depreciation			281,975		281,975	282,805
Excess current expenditures			$ 87,120		$ 87,120	$ 93,242

The accompanying note is an integral part of the financial statements.

MARINE BIOLOGICAL LABORATORY

STATEMENT OF FUNDS

for the year ended December 31, 1973

	Balance December 31, 1972	Gifts and Other Receipts	Investment Income	Used for Current Expenses	Transfers		Other Expenditures	Balance December 31, 1973
					To	From		
Invested funds	$3,806,869	$105,793	$189,050	$160,434	$265,000	$ 920	$82,424	$4,122,934
Unexpended plant funds	—				920		920	—
Unexpended research grants	$ 77,774	577,521		615,308				$ 39,987
Unexpended gifts for designated purposes	$ 87,949	56,862		11,250			9,437	$ 124,124
Current fund	$ 320,852	(87,120) (1)	—			265,000	6,243	$ (37,511)
		$653,056	$189,050	$786,992	$265,920	$265,920	$99,024	
Other gifts and receipts		56,862						
Grants for research, training and support		577,521						
Appropriated from current income and other		105,793						
(1) Excess of current expenditures over income		(87,120)						
		$653,056						
Appropriation for teaching laboratory							920	
Scholarship awards							11,854	
Payments to pensioners							13,077	
Loss on sale of securities							57,493	
Other							15,680	
							$99,024	

The accompanying note is an integral part of the financial statements.

MARINE BIOLOGICAL LABORATORY

SUMMARY OF INVESTMENTS

December 31, 1973

	Cost	*Percent of Total*	*Market Quotations*	*Percent of Total*	*Investment Income 1973*
Securities held by Trustee:					
General endowment fund:					
U. S. Government securities	$ 25,065	1.9	$ 24,800	1.4	$ 1,813
Corporate bonds	595,553	45.0	465,376	26.6	29,025
Preferred stocks	31,094	2.4	17,625	1.0	1,650
Common stocks	670,397	50.7	1,242,107	71.0	41,401
	1,322,109	100.0	1,749,908	100.0	73,889
General educational board endowment fund:					
U. S. Government securities	51,113	16.4	50,592	12.5	3,698
Other bonds	145,965	46.9	109,290	27.0	6,690
Common stocks	114,005	36.7	244,762	60.5	5,824
	311,083	100.0	404,644	100.0	16,212
Total securities held by Trustee	$1,633,192		$2,154,552		90,101
Investments of other endowment and unrestricted funds:					
Pooled investments:					
U. S. Government securities					6,522
Corporate bonds	379,200	40.8	312,908	28.8	13,868
Preferred stocks	62,207	6.7	63,738	5.8	1,120
Common stocks	488,933	52.5	711,709	65.4	22,522
	930,340	100.0	$1,088,355	100.0	44,032
Other investments:					
U. S. Government securities	27,764				1,323
Other bonds	15,029				750
Common stocks	588,016				21,697
Preferred stocks	2,871				104
Real estate	17,550				—
Short-term commercial notes	715,000				38,633
	1,366,230				62,507
Total investments of other endowment and unrestricted funds	$2,296,570				
Total					196,640
Custodian's fees charged thereto					(7,590)
Total investment income					$189,050

Reference: *Biol. Bull.*, **147**: 81–94. (August, 1974)

CALORIFIC VALUES IN THE PHYLUM PLATYHELMINTHES: THE RELATIONSHIP BETWEEN POTENTIAL ENERGY, MODE OF LIFE AND THE EVOLUTION OF ENTOPARASITISM

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An analysis of the calorific values (kcal/g) of seventeen species of animals from six phyla has shown that they have a skewed distribution with a modal frequency at or near the lower range limit (Slobodkin and Richman, 1961). This was regarded as support for the hypothesis that natural selection generally favors production of the maximum number of progeny, rather than a high energy content per unit weight. The latter is favored only sporadically and under special circumstances such as preparation for a period of fasting or stress (Slobodkin, 1962).

To test this hypothesis further we have undertaken a survey of calorific values within the Platyhelminthes. This phylum shows a broad spectrum of life styles which range from the free-living predatory habits of most Turbellaria, through various degrees of facultative or obligate ecto- or entocommensalism (other Turbellaria), to the obligate ecto- or entoparasitism of a few Turbellaria and all Monogenea, Digenea and Cestoda (Jennings, 1971; 1973). The symbiotic species show high fecundity relative to the free-living forms (Hyman, 1951), apparently as an adaptation to their mode of life, and by the above hypothesis could be expected to have correspondingly lower calorific values.

The entosymbiotic species also differ from the free-living ones in that they contain large amounts of carbohydrate, usually in the form of glycogen. This accounts for up to 17.73% of the dry weight in entocommensal turbellarians, 14.77% in digeneans and 26.99% in cestodes, but only 9.96% in free-living flatworms (data from various sources, summarized by Jennings, 1973). The latter, though, generally store lipid and levels of up to 19.83% of the dry weight have been recorded (Jennings, 1957; Mettrick and Boddington, 1972). This dichotomy in the form in which potential energy is stored has long been regarded as stemming from differences in the oxygen tensions of the environments of free-living and entosymbiotic species. Habitats within other organisms, and especially alimentary tracts, often have low oxygen tensions (Campbell, 1931; von Brand, 1946; Rogers, 1949; Crompton, Shrimpton and Silver, 1965) and in such situations carbohydrates may well be the most appropriate metabolic substrates. There is certainly much evidence, ably reviewed by Read (1968), that many parasitic flatworms have metabolic processes suited to life under semi-anaerobic conditions. However, as Crompton, *et al.*, 1965 emphasize, the partial pressure of oxygen in regions favored by entosymbiotes still usually exceeds the value of 5 mm Hg given by Hill

(1936) as the threshold necessary for satisfactory functioning of cytochrome oxidase. Further, some entosymbiotes live in fully aerobic situations and yet store glycogen (Halton, 1967; Mettrick and Jennings, 1969), so that this phenomenon cannot be linked directly with oxygen tension. Recognizing this, Jennings and Mettrick (1968) have suggested that glycogen storage, instead, is linked in some way with high fecundity. Carbohydrates, having lower calorific values than lipids (Cummins and Wuychek, 1971), may reduce whole body values for the entosymbiotes and this notion therefore conforms to the hypothesis cited by Slobodkin and Richman (1961) that fecundity generally implies a low calorific value.

Consequently, in addition to testing the general applicability of this hypothesis, by examining calorific values in the phylum Platyhelminthes, we have also examined the validity of its particular implications for entosymbiotic animals. This has been done by selecting species for examination which are representative of the various life styles in this phylum so that any correlation between potential energy and mode of life would be revealed.

Material and Methods

The species investigated are listed systematically together with some details of their habitats and modes of life. The few species for which calorific values have already been published are also listed, with the authority, since the data for these will be incorporated with those from the present study.

Turbellaria

Rhabdocoela

Syndesmis franciscana (Lehmann). Entocommensal in the gut and coelom of *Strongylocentrotus purpuratus* Clark (Echinoidea), from Bodega Bay, California.

Allococoela

Plagiostomum sulphureum von Graff. Free-living, marine littoral, Filey Brigg, Yorkshire, England.

Tricladida

Bdelloura candida (Girard). Ectocommensal on the gills and limbs of *Limulus polyphemus* L. (Xiphosura), Cape Cod, Massachusetts.

Dendrocoelum lacteum (O. F. Müller). Free-living, freshwater littoral, Loch Lomond, Scotland.

Dugesia lugubris (O. Schmidt). As *D. lacteum*.

Planaria torva (O. F. Müller). As *D. lacteum*.

Polycelis nigra (O. F. Müller). Free-living, freshwater littoral, Malham Tarn, Yorkshire, England.

Procerodes ulvae (Diesing). Free-living, marine littoral, Isle of Cumbrae, Scotland.

Bipalium kewense Moseley. Free-living, terrestrial. Data from Slobodkin and Richman, 1961.

Dugesia tigrina (Girard). Free-living, freshwater littoral. From Slobodkin and Richman, 1961.

Phagocata gracilis (Haldeman). As *D. tigrina.* From Minshall, G. W., unpublished data, cited in Cummins and Wuychek, 1971.

Phagocata morgani (Stevens and Boring). As *D. tigrina.* From Teal, 1957.

Phagocata woodworthi Hyman. As *D. tigrina.* From Teal, 1957.

Monogenea

Polyopisthocotylea

Diclidophora merlangi (Kroyer). Ectoparasitic on the gills of the whiting *Gadus merlangus* (L.) (Teleostei), Whitby, Yorkshire, England.

Entodbella soleae Johnston. Ectoparasitic on the skin of the sole *Solea solea* (L.) (Teleostei), Plymouth, England.

Polystoma integerrimum Rudolphi. Entoparasitic in the urinary bladder of *Rana temporaria* L. (Amphibia), Adel, Yorkshire, England.

Digenea

Prosostomata

Fasciola hepatica L. Entoparasitic in the biliary system of domestic sheep *Ovis aries* L. (Mammalia), slaughtered in Glasgow, Scotland.

Haplometra cylindracea Looss. Entoparasitic in the lungs of *Rana temporaria* L. (Amphibia), Adel, Yorkshire, England.

Cestoda

Cyclophyllidea

Hymenolepis diminuta Rudolphi. Entoparasitic in the intestine of laboratory rats, *Rattus norvegicus* Berkenhout, Wistar strain (Mammalia), Department of Zoology, University of Toronto.

Pseudophyllidea

Triaenophorus nodulosus Pallas. Entoparasitic in the intestine of the pike *Esox lucius* L. (Teleostei), R. Ouse, Yorkshire, England.

Tetraphyllidea

Echeneibothrium variable Van Beneden. Entoparasitic in the spiral valve of the skate *Raia clavata* L. (Elasmobranchii), Whitby, Yorkshire, England.

The four freshwater species of triclads (*D. lacteum, D. iugubris, P. torva* and *P. nigra*) were kept in the laboratory for two weeks prior to examination and fed daily on the oligochaete *Tubifex*, apart from one subgroup of *P. nigra* which were starved for this period. All other species were processed immediately after collection from the field, or removal from their hosts.

Calorimetry

Living healthy specimens were rinsed briefly in distilled water and then either freeze dried to constant weight (*Syndesmis franciscana* and *Hymenolepis diminuta*)

TABLE I

Calorific values (kcal/g ash-free dry wt), Joule equivalents and ash contents of free living and symbiotic Platyhelminthes

Species and mode of life	kcal/g ash-free dry wt ± 95% conf. interval	kJ g ash-free dry wt	Ash % dry wt ± 95% conf. interval**	Number of measurements
Free living				
D. lacteum	6.316 ± 0.317	26.439	5.82 ± 0.39	10
D. lugubris	6.295 ± 0.222	26.351	3.50 ± 0.27	10
P. nigra	6.420 ± 0.338	26.874	6.10 ± 0.71	10
P. sulphureum	6.798†	28.456	7.71 —	1
P. torva	6.382 ± 0.451	26.715	3.79 ± 0.42	10
P. ulvae	6.000 ± 0.258	25.116	6.95 ± 0.72	3
B. kewense	5.684 ± 0.124	23.793	*	*
D. tigrina	6.286 ± 0.338	26.313	*	*
P. gracilis	6.377 ± 0.137	26.694	*	*
P. morgani	5.600†	23.442	*	*
P. woodworthi	5.600†			
Mean	6.216 ± 0.258	26.019	5.65 ± 0.41	
Ectocommensal				
B. candida	5.897†	24.685	10.09†	1
Entocommensal				
S. franciscana	5.080 ± 0.216	21.265	11.72 ± 2.35	3
Ectoparasitic				
D. merlangi	5.372†	22.487	8.98†	1
E. soleae	5.668†	23.726	6.10†	1
Mean	5.520	23.107	7.51	
Entoparasitic (except Cestoda)				
F. hepatica	5.205 ± 0.201	21.788	5.07 ± 0.37	10
H. cylindracea	5.124†	21.449	6.53†	1
P. integerrimum	5.372†	22.487	5.98†	1
Mean	5.234 ± 0.292	21.908	5.85 ± 0.15	
Cestoda				
E. variable	5.164 ± 0.316	21.616	3.86 ± 0.39	5
H. diminuta	5.817 ± 0.203	24.350	4.30 ± 0.41	10
T. nodulosus	5.972†	24.999	2.17†	1
Mean	5.651 ± 0.561	23.655	3.44 ± 0.67	
Grand mean for all species	5.841 ± 0.230	24.452	6.17 ± 0.08	

† = insufficient material for replicates.

* = data from other authors, % ash and number of measurements not specified.

** = data were transformed to arcsines before calculation of mean and confidence intervals, and have been retransformed for presentation (Rohlf and Sokal, 1969).

or air dried at 60° C for twenty four hours. The dried specimens were ground into powder and calorific values determined for the entire animal. In *H. diminuta* individuals were also divided into three portions, consisting of immature, mature and gravid proglottids and each portion was then processed separately to detect any differences in energy reserves related to the different reproductive states.

Calorific values were determined in a Phillipson micro-bomb calorimeter using standard calorimetric techniques (Phillipson, 1964). Each sample used was of at least 10 mg dry weight and usually was in excess of 15 mg. Routine corrections were applied for fuse wire "glow" and acid production (Paine, 1971). The ash contents were usually less than 10% of the dry weight (only four samples had > 10% ash) and consequently no correction was applied for endothermy (Paine, 1966). Due to the small size of most of the specimens and the difficulties of obtaining suitable numbers there was not, in most cases, enough material for independent determination of ash content in a muffle furnace. This information was routinely obtained, therefore, from the residues remaining in the bomb after firing. Data were discarded if fuse wire contamination was suspected.

Replicate samples were used whenever the amount of material available permitted. When possible, up to ten subsamples were examined, means were computed together with fiducial limits and the latter were corrected for small sample sizes (*i.e.*, $t.\frac{s}{\sqrt{n}}$, where t was obtained at the 95% probability level for $n - 1$ degrees of freedom). Means for species with common modes of life were then averaged. Data were compiled in this fashion because it was considered that the resultant means and variances were of greater importance than those based on individual measurements.

Calorific values for free-living triclads obtained by previous investigators were used either directly (*e.g.*, those for *B. kewense* and *D. tigrina* given by Slobodkin and Richman, 1961), or after adjustment to make them comparable with those obtained here. The values given by Teal (1957) for two species of *Phagocata* were obtained by dichromate digestion and expressed in terms of wet weight. To obtain an ash-free dry weight estimate it has been assumed, from experience in drying samples, that dry weight equals 25% of wet weight and that ash equals 5% of dry weight (the average ash value for the four freshwater triclads studied here being 5.038% as calculated from the data in Table I).

RESULTS

The potential energy contents of the 21 species of flatworms investigated are listed in Table I, together with their ash contents expressed as a percentage of the dry weight. Energy values are given in terms of kcal/g ash-free dry weight and S. I. (Joule) equivalents are also shown. Fiducial limits on the calorific values ranged between 2.14–7.06% of the means, which is generally considered to be acceptable for biological samples.

Before considering these results in detail it should be pointed out that, apart from random manipulative errors, two further factors may have influenced the data. First, no correction has been made for nitrogen production during combustion. This may have resulted in an underestimate of the calorific values, but since there appears to be little interspecific variation in nitrogen contents within the Platyhelminthes (Mettrick and Boddington, 1972) comparative treatment of the data is still legitimate (Kersting, 1972). Secondly, bomb combustion, rather than muffle furnace estimates of ash, can introduce systematic errors into calorific

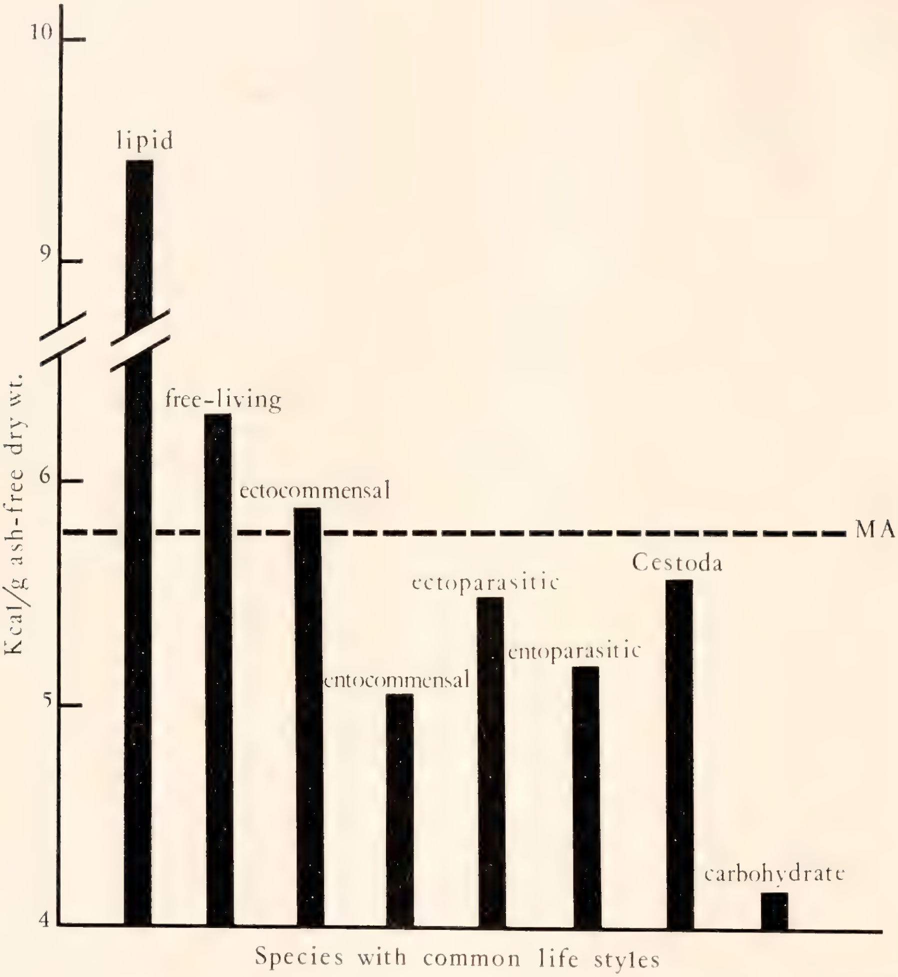

FIGURE 1. Mean calorific values (kcal/g ash-free dry weight) of flatworms with common life styles. The values for lipid, carbohydrate and MA (the mean value for whole animals from other phyla) are from Cummins and Wuychek (1971).

values. However, this depends on the ash content of the materials involved and becomes more extreme as percentage ash increases. At ash levels below 10% (most of the species considered here) the effect becomes trivial and contributes less than ± 1% variation (Reiners and Reiners, 1972).

The grand mean of 5.841 ± 0.230 kcal/g ash-free dry weight for all the species studied falls close to the mean value of 5.821 kcal/g ash-free dry weight previously reported for whole animals from a wide range of other phyla (data summarized by Cummins and Wuychek, 1971). Values ranged from a minimum of 5.080 kcal/g ash-free dry weight for the entocommensal *S. franciscana* to a maximum of 6.798 kcal/g ash-free dry weight for the free-living *P. sulphureum.*

The mean calorific values for species sharing common life styles are summarized in Figure 1. This shows that the free-living flatworms have a value higher than the mean for whole animals from other phyla (MA) whereas the entocommensal and entoparasitic species, other than cestodes, have much lower values. The ectocommensal, ectoparasites and cestodes, however, have values very close to this mean.

The differences in calorific values and ash contents between the three regions of the cestode *H. diminuta* are shown in Table II, together with values for the whole worm. The calorific values of immature and mature proglottids are similar to each other, but significantly smaller than that of the gravid proglottids ($d = 2.53$, $P \simeq 0.02$). The value for the whole worm is only slightly less than that of the gravid component and is obviously elevated by this. The immature and mature proglottids are more comparable to the whole flatworms of other classes, in terms of growth and reproductive activity, than are the gravid proglottids and the mean calorific value for these two regions (5.554 kcal/g ash-free dry weight) is somewhat closer to those for the entocommensal and entoparasitic species than is the value for whole cestodes.

Figure 1 also shows average calorific values for pure lipids and carbohydrates. The range between these two extremes represents that which is theoretically possible in organic material but the actual range recorded here within the Platyhelminthes is considerably less. This is also the case in all other organisms so far studied (Cummins and Wuychek, 1971).

Starvation for two weeks caused a significant fall in the calorific value of the free-living triclad *P. nigra.* The value dropped from 6.420 kcal/g ash-free dry weight to 5.050 kcal/g ash-free dry weight, and the ash content fell from 6.10% dry weight to 5.98%. The average size decreased by approximately 30%.

Discussion

Calorific values in the Platyhelminthes follow the general pattern observed in other phyla in that they are skewed towards the lower possible limit (*i.e.* the value for carbohydrate, Figure 1), with a mean of 5.841 kcal/g ash-free dry weight and a range of 5.080–6.798 kcal/g ash-free dry weight. The distribution within this range, however, is not haphazard but shows a definite relationship to the mode of life. Entosymbiotic species, with the exception of the Cestoda, have values which cluster towards the lower limit of the range and are generally less than the mean level for animals from other phyla (MA of Figure 1 and referred to hereafter as "mean animal"), whereas values for the free-living flatworms cluster toward a higher level and have a mean in excess of that for the "mean animal." The ectosymbiotes and cestodes are intermediate between these two extremes.

Fluctuations in ash-free calorific values around the "mean animal" level have been identified almost exclusively with fluctuations in tissue lipid concentration (Ostapenya and Sergeev, 1963). This is because proteins and carbohydrates have similar energy equivalents (*circa* 5.7 and 4.1 kcal/g ash-free dry weight, respectively), while lipids have far greater equivalents (*ca.* 9.5 kcal/g ash-free dry weight). Thus our results confirm that the evolutionary trend within the Platyhelminthes, assuming that the free-living forms are primitive, has been toward a reduction in the amount of lipid stored per unit weight with a concomitant reduction in calorific values.

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The cestodes are somewhat atypical, when compared with the other entosymbiotes, in that they have relatively high and variable calorific values (Table I). The latter probably result from variability in lipid contents; high lipid levels have been reported in *Hymenolepis diminuta* by Webb and Mettrick (1973) but much lower levels are recorded for several other cestodes (Smyth, 1966). In *H. diminuta* gravid proglottids contain a higher proportion of lipid than do immature and mature ones (Fairbairn, Wertheim, Harpur and Schiller, 1961; Mettrick and Cannon, 1970) and this is reflected in their high calorific value (Table II). If the cestode strobila is viewed as a series of progressively maturing reproductive units, though, rather than as a single individual, it becomes legitimate to compare only the immature and mature proglottids with the whole individuals from other flatworm groups. The gravid proglottids contain the products of reproduction and are more comparable, therefore, to the cocoons of free-living flatworms. The immature and mature proglottids have calorific values closer to those of other entosymbiotes than does the whole cestode (Table II) and so conform more closely to the basic evolutionary trend in the phylum.

TABLE II

Calorific values (kcal/g ash-free dry wt.), Joule equivalents and ash contents in Hymenolepis diminuta

	kcal/g ash-free dry wt ± 95% conf. interval	kJ/g ash-free dry wt	Ash % dry wt ± 95% conf. interval	Number of measurements
Whole worm	5.817 ± 0.203	24.35	4.30 ± 0.41	10
Immature proglottids	5.559†	23.27	3.30†	1
Mature proglottids	5.550 ± 0.241	23.23	3.54 ± 0.39	8
Gravid proglottids	5.997 ± 0.185	25.10	3.35 ± 0.28	10

† = insufficient material for replicates.

The link between calorific value, fecundity and mode of life would seem, therefore, to be amply demonstrated in the Platyhelminthes. The precise nature of this link is at first difficult to define, but the situation becomes clearer when it is recognized that "survival of the fittest" must be interpreted in terms of the survival rate of offspring rather than entirely in terms of survival of the adult. Thus the "fittest" individual is one which produces the greatest number of viable offspring, and selection will operate in favor of using any energy available in excess of growth and maintenance requirements for progeny production rather than for accumulation of endogenous food stores. In Slobodkin's words "there is a selective advantage in increasing fecundity but not adiposity" (Slobodkin, 1962, page 71). From this viewpoint it is the free-living flatworms which are atypical in the Platyhelminthes and the onus is put on explaining their high calorific values, resulting from lipid stores, rather than on explaining the low energy equivalents, resulting from glycogen stores, in the entosymbiotes.

Lipid has the advantage over glycogen as a long-term energy reserve in that it allows more calories to be stored per unit weight of tissue or, conversely, less weight need be added per unit energy stored. Energy storage is essential in species likely to experience prolonged periods of hardship, and lipid accumulation with concomitant increase in calorific value occurs, for example, in birds (Odum, Marshal and Marples, 1965) and termites (Wiegert and Coleman, 1970) prior to migration. It has also been suggested that lipid storage is an adaptation for locomotion, because of the favorable weight/potential energy ratio (Stetten and Stetten, 1956) and this argument could be applied in the case of the free-living flatworms. The principal cause, though, behind the transformation of excess energy into lipids in the free-living species must be that it increases the chances of progeny survival by allowing individuals to survive poor feeding conditions and then subsequently breed. This strategy is obviously advantageous in iteroparous organisms like the triclad Turbellaria, which breed more than once in their life span. Here, survival of adults that might already have bred is equally as important as survival of juveniles. This is in contrast to semelparous organisms, breeding only once in the life span, where juvenile survival may be more important than preservation of adults that might already have bred (Calow, 1973). Some acoel and rhabdocoel turbellarians are semelparous and it would be interesting to compare lipid and glycogen contents and calorific values in these at various stages in the life history. In freshwater molluscs, however, which are similarly semelparous glycogen storage predominates and in fact increases in preparation for over-wintering (Goddard and Martin, 1966). These animals, though, are slow-moving, which permits the storage of less potential energy per unit weight (*vide* Stetten and Stetten, 1956), and they are also herbivorous. This habit, as opposed to the carnivorous one, reduces the probability of either prolonged or intense starvation (Hairston, Smith and Slobodkin, 1960) so that long term energy stores, *i.e.* lipid, are less important.

The triclads utilize lipid during starvation (Jennings, 1957; Boddington and Mettrick, 1971) and, as shown in the present study, this is accompanied by a decrease in calorific value. The importance of this ability, coupled with the triclad's capacity to resorb its tissues and then grow again when conditions permit, has been demonstrated in a classic study by Reynoldson (1966). Reynoldson showed that hatching of the eggs, in European triclad populations, is followed by gradual development of food shortage as the young grow. This slows and eventually stops breeding. The triclads then decline in size, resorbing their tissues, and this is accompanied by some mortality which readjusts population size to food supply. The survivors are then able to feed, grow and reproduce in a further cycle, as conditions permit.

The symbiotic flatworms, and especially the parasitic species, are buffered from such variations in food supply. Ectocommensals are perhaps the least buffered, for their nutritional physiology resembles that of free-living species (Jennings, 1968, 1973). In the species studied here the calorific value is higher than the mean for the ectoparasites (Fig. 1), which have a more stable food supply, but it is still lower than that of the free-living species. In entosymbiotic species, as in the ectoparasites, the food supply is relatively stable. The entocommensals feed on protozoa and other small organisms which are either co-commensals or components of the host's food; in both cases they are abundant and readily available to the entocommensal flatworm (Jennings, 1973). In the entoparasitic species the stability of the food supply is self-evident for tissue-feeders and sanguivores (Monogenea and Digenea), and this is also the case for the

Cestoda which live, almost without exception, in the intestine of vertebrates. Here the host actively maintains a constant level of amino acids, vitamins and other organic substances in the gut lumen, by means of the exocrine-enteric circulation (Read, 1950; Read, Simmons, Campbell and Rothman, 1960; Nasset and Ju, 1961; Nasset, 1965; Read, 1970), and this will buffer the cestode from any temporary changes in the quantity or composition of the host's ingesta.

The adoption of an entosymbiotic life style, therefore, removes the need for long-term storage products because it makes available a stable source of food. The entosymbiote is freed from the need to form lipid reserves to ensure survival until it can breed and we suggest that this is the prime reason for the reduced calorific values of entosymbiotic flatworms. These reduced values may also be related to the fact that entosymbiotic species, once mature, tend to breed more or less continuously throughout their life span and thus will be continually exporting lipid as a component of the eggs. Free-living species, whether semelparous or iteroparous, do not have this prolonged reproductive phase and their relatively high lipid content could be due to a build-up of lipid which is subsequently exported during reproduction. A comparable situation is seen, for example, in freshwater animals which accumulate inorganic salts before breeding for incorporation in the eggs. This hypothesis cannot be tested, as yet, due to lack of appropriate data. We suggest, though, that even if it were proved to be correct it could account for only a proportion of the discrepancy between the calorific values of symbiotic and non-symbiotic flatworms, and that reliability of the food supply is the major factor.

The food supply of entoparasites would seem to be even more reliable than that of entocommensals, and so the evolution of entoparasitism is seen as a logical conclusion to the tendency to form symbiotic associations which is a dominant feature of the biology of the Platyhelminthes.

The entosymbiotic life style, though, places a very considerable premium on production of offspring, because of the problems of dispersal between hosts. This can be met by using resources that in free-living flatworms would have to be diverted into lipid reserves. Such increased production of progeny presumably involves considerable expenditure of energy and since long-term storage is not involved glycogen rather than lipid is the most suitable substrate. It is readily metabolized and its high molecular weight and low intrinsic viscosity permit accumulation in tissues without much effect on osmotic pressure (Stetten and Stetten, 1956). Thus, although there may be fairly rapid turn-over of glycogen in entosymbiotes that are continually producing eggs, its properties are such that the tissues can always contain relatively large amounts.

The occurrence of large quantities of glycogen in entosymbiotes appears, therefore, to be basically an adaptation for high fecundity, necessitated by the mode of life, and is not primarily an adaptation related to environmental oxygen tension, as has been previously suggested. Nevertheless, as pointed out by Jennings (1973), the presence of glycogen in entosymbiotes does constitute a pre-adaptation for life under conditions of low or variable oxygen tension, in that a suitable substrate is already available for partially or totally anaerobic respiration. A number of entosymbiotic flatworms, notably the Cestoda, have exploited this potential and colonized habitats such as the vertebrate intestine where the oxygen concentration may be much reduced. In such species there is, without doubt, a direct relationship between their high glycogen content, the low environmental oxygen tension, and their modified respiratory physiology, but on the above argument this relationship is seen to be proximate rather than ultimate in nature.

An interesting consequence of our interpretation of the relationship between the nature of the food reserve, mode of life and level of fecundity, is that parasitism, and especially entoparasitism can be regarded simply as adaptive devices which favor high fecundity. This view, of course, is the reverse of the classical view, already cited, that high fecundity is a basic and essential adaptation for the parasitic habit.

In explaining the distribution of calorific values within the Platyhelminthes we have emphasized the importance of reproductive strategy and food availability. This is because groups with different life styles, ranging from completely parasitic to completely free-living, fit exactly into the distribution spectrum from low to high caloric values and we believe that the only parameters which show the same distribution are fecundity and food availability. We dismiss the direct involvement of oxygen tension for reasons already stated but accept that mobility might be of some importance. For example, free-living forms may need to be more mobile than parasites and thus might require to store more energy per unit weight. This could account for their higher calorific values. However, such an argument presupposes that food stores are necessary anyway, and in this sense is less basic than our explanation in terms of fecundity and food supply. Furthermore, the two possibilities are not mutually exclusive.

There may, of course, be more subtle explanations. For example, there could be some biochemical disadvantages (other than those stemming from reduced oxygen tension) associated with lipid storage, and hence high calorific values, in the enclosed entosymbiotic environment. It is difficult, though, to envisage what these could be and so we present our argument as the most plausible within the limits of currently available information.

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SUMMARY

1. A comparison has been made of the calorific values (kcal/g ash-free dry weight) of 21 species of flatworms which exhibit, between them, the various life styles found in the phylum Platyhelminthes.

2. The grand mean calorific value for whole animals of all 21 species was 5.841 ± 0.230 kcal/g ash-free dry weight, which is close to the mean of 5.821 kcal/g ash-free dry weight reported for whole animals from a wide range of other phyla.

3. There is a direct relationship between mode of life and calorific value. Free-living flatworms have a mean value higher than the mean for whole animals in general, while entocommensal and entoparasitic species other than cestodes have

lower values. The ectocommensal, ectoparasites and cestodes have values close to the mean.

4. In the cestode *H. diminuta* the values for immature and mature proglottids (regions comparable to whole flatworms from other classes) are less than the mean for whole animals from other phyla and approach those for the entocommensal and entoparasitic flatworms. Gravid proglottids have a higher mean.

5. The entosymbiotic flatworms, with high fecundity relative to free-living species, thus conform to the hypothesis that fecundity is linked with low potential energy per unit weight.

6. The dichotomy in food reserves in the Platyhelminthes, with storage of lipid in free-living species and of carbohydrate in the symbiotic forms is considered, therefore, to be basically an adaptation to different reproductive patterns necessitated by different life styles.

7. It is suggested that the emphasis on carbohydrate as an energy source, rather than lipid, pre-adapted entosymbiotes for partial or total anaerobic respiration. The relationship between high glycogen content, low environmental oxygen tension and modified respiratory physiology occurring in some entoparasites is thus proximate rather than ultimate in nature.

8. The evolution of entoparasitism is seen as a logical conclusion to the trend toward the formation of symbiotic relationships, which is a characteristic feature of the Platyhelminthes, in that it removes the need for long-term reserves and thereby allows total emphasis on progeny production. It follows that the parasitic habit, and especially entoparasitism, provides conditions necessary for high fecundity so that the latter can be regarded as a direct and automatic consequence of the parasitic mode of life, rather than as a specific adaptation to it.

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LOCOMOTION OF THE HOLOTHURIAN *EUAPTA LAPPA* AND REDEFINITION OF PERISTALSIS

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Observation of the holothurian *Euapta lappa* (J. Muller, 1853) reveals locomotory waves that are unlike any previously described. According to the terminology of Vles (1907), the wave is direct rather than retrograde, meaning that it moves in the same direction as the animal—in this case, anteriorly. In *Euapta,* the wave is peristaltic and involves no elevation of the body cylinder's major axis. Direct peristaltic locomotory waves, previously described in the holothurians *Stichopus panimensis* and *Astichopus multifidus* have an arch above the substratum formed by the localized temporary elevation of the body cylinder's major axis (Parker, 1921; Glynn, 1965). Although retrograde waves in wormlike animals are well understood (Gray and Lissman, 1938; Clark, 1964), direct locomotory waves are less well known (Elder, 1973a, 1973b). This paper presents the first description of locomotion in *Euapta* and a resolution of the ambiguity of the term "peristalsis." "Peristalsis" is herein redefined as any muscular contraction moving along a radially flexible tube in such a way that each component wave of circular, longitudinal or oblique muscular contraction is preceded or followed by a period of relative relaxation of all similarly oriented muscle within a given tubular segment. A classification of some known types of peristalsis is also presented.

THE EXPERIMENTAL ANIMAL AND METHODS

Euapta lappa is an apodous holothurian with a wormlike appearance. The animal has an extremely flexible body which may attain an extended length of 1 m and a diameter of 2–4 cm. If disturbed, however, *Euapta* can contract regions of the body to about one third their extended lengths. The anterior end has a circumoral ring of 15 pinnate tentacles and the body cylinder, in a condition of tonus (resting muscular tension), has five radially symmetric rows of outpocketings called warts (Fig. 1). The animal has both circular and longitudinal muscles; the former are a continuous tube throughout the body; the latter are restricted to 5 strips arranged in radial symmetry and extending the length of the body. The animal's body surface is covered with many anchor-shaped calcareous ossicles (Hyman, 1955) approximately 0.4 mm long which aid in attachment to the substratum.

Specimens were obtained from Tropical Atlantic Marine Specimens, Big Pine Key, Florida, and kept in a 25 gallon aquarium filled with recirculated artificial sea water ("Instant Ocean") on a crushed shell substratum. Observation was the main technique and a continuous-feeding 35 mm movie camera (Grass) was used to record and "slow down" the motion. The camera's shutter was always open and images were formed by the flashing of a stroboscopic light in a dark room. For filming, the animal was transferred to a smaller and narrower tank provided with a centimeter grid as a background for reference. Living body wall was examined with a Wild M-5 dissecting microscope with polarizing attachments.

RESULTS

Warts (Fig. 1) are not permanent structures. Individual warts may be observed to shift or "slide" slightly along the major body axis, to increase or decrease in size and to divide into two smaller warts or merge to form a larger wart. Warts do not divide or merge circumferentially. A single wart will collapse when its apex is pinched with forceps. Animals anesthetized with magnesium salts have a body cylinder of greater average diameter with no warts.

Histological preparations failed to reveal any morphologically distinct structure that might account for the warts. In living animals, a number of warts were tagged by threading them with a suture. But subsequent vivisection did not reveal any distinct correlated structures except that tags were always between two adjacent strips of longitudinal muscle (Fig. 1a). The five relatively narrow strips of longitudinal muscles are separated by five relatively wider strips of body wall lacking longitudinal muscles. This pattern of longitudinal muscle is superimposed on the continuous circular muscle layer.

Thus, we conclude that warts in *Euapta* and probably those in other similar apodous holothurians are not specific morphologically distinct points or structures as is suggested by other writers (Hyman, 1955). Instead, warts appear to be areas of limited contraction of circular muscle bounded by regions of greater degree of contraction of circular muscles. These holothurian warts then are analogous to the haustrations of the vertebrate large intestine (Guyton, 1971). Divisions of warts are effected by contraction of additional circular muscles, and merging occurs when a contracted ring of circular muscle relaxes.

The locomotory wave (Fig. 1b)

By observing the distance between points on the body wall, it is possible to tell when longitudinal or circular muscles are contracting. As the direct locomotory wave passes along a section of the body wall, contraction of longitudinal muscles (B) is the first evident occurrence. Warts and recognizable pigmented points on the body wall come closer together longitudinally in an accordion-like effect. Then, while longitudinal contraction is still under way, circular contraction occurs (C) as is evidenced by the decrease in diameter of the body cylinder and the "flattening" of warts. This is followed by relaxation and extension of all body wall musculature (D) so that the entire body wall bulges outward. Expansion is follower by a return to the original tonus (E).

Each locomotory wave starts at the posterior end of the animal by the same sequence of longitudinal and circular contraction. Each part of the body wall advances while under longitudinal contraction. The major axis of the body is not raised in the formation of an arch but rather an arch is formed by a decrease in diameter of the body cylinder. The body axis does not change its position. Con-

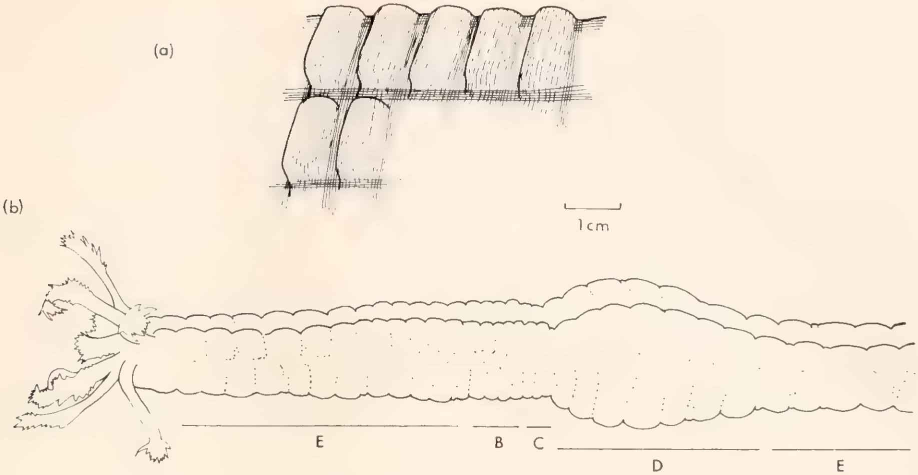

FIGURE 1. Diagrams showing the external shape of the apodous holothurian *Euapta lappa.* (a) Seven warts are shown with the muscles that delineate them: solid lines indicate muscles in state of tonus; broken lines indicate relaxed circular muscles; (b) body of *Euapta* with a direct overlapping peristaltic wave passing from right to left; B, initial contraction of longitudinal muscle; C, contraction of circular muscle and further longitudinal muscle contractions; D, relaxation of all muscles: body wall distended by coelomic pressure; E, state of tonus.

traction of circular muscles releases the body wall from its attachment to the substratum, thus facilitating forward movement.

The *direct overlapping peristalsis* described above is the most common method of locomotion in *Euapta,* but variations do exist as a result of the reduction or complete suppression of some of its components. One type of wave, *direct longitudinal peristalsis,* is characterized by longitudinal muscular contractions as observed in the direct overlapping peristalsis, but there is no accompanying wave of circular contraction. Direct longitudinal peristalsis is observed when the animal crawls across smooth glass surfaces and when it is climbing up a vertical aquarium wall clinging by its tentacles with part or all of its body hanging free below. Sometimes this direct longitudinal paristalsis passes along a body cylinder which is in a tonus state of circular contraction. At other times the body cylinder is constricted throughout part or all of its length by continuous contraction of circular muscles. This obliterates all warts and holds the body diameter constant while waves of only longitudinal contraction pass along the body. In these two cases of waves of longitudinal muscle contraction passing along a body cylinder of unchanging diameter, it is clear that friction is minimal and offers little help or hindrance to forward motion. Direct longitudinal peristalsis is further considered in the discussion.

Another type, *direct overlapping partially circular peristalsis,* has normal contraction of longitudinal muscle but only the two ventral-most warts are retracted, as opposed to the full circumferential contraction by circular muscles seen in direct overlapping peristalsis. This wave of incomplete circular muscle contraction was observed on only two occasions and was never recorded on film.

There is another wave which may be of interest. Viewed infrequently while the animal is resting, this *circular contraction peristalsis* is a wave of contraction of only circular muscles that seems to have no locomotory function.

These different waves are not necessarily mutually exclusive behavioral events. A wave in *Euapta* may start as a certain type; by the time it reaches the anterior end it may be modified to one of its variations. Intermediate forms exist.

Upon first viewing *Euapta* move by direct overlapping peristalsis, it may be apparent why longitudinal contraction advances a more posterior point rather than pulling an anterior point posteriorly. Obviously circular contraction raises the body wall from the substratum while the still-attached body wall anterior to the locomotory wave acts as a fixed point of attachment. Thus, longitudinal contraction will move detached points forward toward the fixed point. In addition, *Euapta's* calcareous ossicles are believed to be so situated that they catch the substratum when pulled posteriorly and release their hold when pulled anteriorly (Hyman, 1955). It appears that opportunities for catching onto the substratum are maximum when the body is maximally inflated and the epidermis is stretched tightly over the ossicles. Portions of the body anterior to a locomotory wave are under slight longitudinal stretch which is increased by the approaching longitudinal contraction. Accordingly, these regions, just anterior to a locomotory wave, will have their ossicles well protruded and attached to the substratum, each forming a fixed point. During locomotion the tentacles also act as fixed points at the anterior end. Each tentacle in turn sweeps from its extended position in front of the animal, backward toward the mouth. It then reaches forward and attaches to the substratum creating a fixed point. A tentacle may subsequently detach and sweep back toward the mouth or it may first contract longitudinally and take up any "slack" left by a peristaltic wave before detaching.

Discussion

When *Euapta* hangs from the vertical glass wall of an aquarium, the tentacles are the only fixed points of attachment. In this situation direct longitudinal peristalsis continues to serve a locomotory function. Each such wave gives the animal a boost as it climbs the glass. Each wave begins by pulling the posterior end upward and forward. The region of longitudinal compression thus formed is propagated anteriorly. This situation is analogous to that of a "slinky" toy, hanging vertically from one end. A "slinky" toy is a long easily extensible spring with a small spring constant, K, according to the equation $F = -KX$ where F is the force generated by the spring and X is the linear displacement of an end of the spring stretched from its resting length. Such springs hung vertically will elongate to many times their resting length due to gravity alone.

If the lower end of a vertically hung "slinky" is pushed up one foot and released, a wave of compression is formed which travels upward while the lower end of the spring begins to fall back down. The upward travelling wave of longitudinal compression, upon reaching the upper end, provides an upward force opposing the downward force of gravity. Meanwhile, as the lower end which was lifted up and then released begins to fall back down, the downward force of gravity acting upon the mass of the spring at the lower end is manifest in the motion of the falling segment of spring. Thus, as long as the lower part of the spring is still falling, the weight of this lower part of the "slinky" is not borne by a point of attachment at the upper end. However, the lower end of the spring is brought to an abrupt stop and would need to be stretched to continue any further downward motion. The point of attachment now must provide an upward force equal and opposite to the force of the downward acceleration of gravity acting upon the mass of the lower end of the spring; the point of attachment reassumes support of the weight of the lower end of the spring. In addition, the fixed point of attachment must momentarily supply another upward force: one that is not even required when the fixed point is supporting the whole body weight. This upward force is needed to cause the rapid deceleration (an upward acceleration) of the falling mass of the lower end of the spring as it comes to a stop.

In the instance of *Euapta* hanging and climbing by its tentacles, a wave of longitudinal compression reaches the anterior end, providing an upward force. This force causes an upward displacement of the anterior end. The tentacles for a moment do not have to support the full weight of the body. While some of the tentacles maintain their hold, others reach forward and secure a new hold before they must resume full support of the body mass and absorb the momentum of the falling lower body region.

Definitions of "peristalsis" found in dictionaries are contradictory and confusing. We have given a description of the direct peristaltic locomotory waves found in *Euapta*, which are very different from the direct peristaltic locomotory waves described for *Astichopus* by Glynn (1965). Parker (1921) did not use the term "peristalsis" to describe the wave he observed in *Stichopus* but accounts of his work by text writers (Clark, 1964) indicate that the wave he observed is considered by them to be peristaltic. Elder (1973b) states that locomotion in the apodous holothurian *Leptosynapta* is achieved by means of direct peristaltic waves involving simultaneous longitudinal and circular muscle contraction. Duncan and Pickwell (1939, page 141) described the telescopic locomotion of dipteran larvae as being "similar to peristalsis." A more recent reviewer, (Hughes, 1965, page 233) however considers apodous larvae to be "entirely dependent on peristaltic movements of the body for propulsion." Direct pedal locomotory waves of gastropods and the direct peristaltic locomotory waves of *Euapta* both function by similar mechanisms. In each case the ventrum is lifted from the substratum and then advanced by contraction of longitudinal muscles. Yet the latter is termed peristaltic and the former is not. All too often an author, upon identifying a wave as peristaltic, believes he has adequately characterized the wave. Some authors (Code and Carlson, 1968; and Ritchie, Truelove, Ardran and Tuckey, 1971) working with mammalian intestinal contractions; seem to have adopted the definition of peristalsis as a propulsive wave of contraction of part or all of the circular muscle. This working definition, however, excludes contractions observed in invertebrates which have long been termed peristaltic.

Mammalian gastroenterologists have what is perhaps an even more confusing terminology problem. Balloons and open-tipped catheters inserted into the mammalian gastrointestinal tract yield four distinct types of pressure wave recordings. These recordings include pressure changes caused by peristaltic as well as nonperistaltic contractions. Two of these four classifications differ only in the amplitude of their fluctuation (Hightower, 1968). There is no clear accepted correlation between types of pressure recordings and the varied types of gastrointestinal movement recognized by direct observation and cineradiography; segmenting movements, pendulum movements, peristalsis and peristaltic rushes. Some authors believe that the pendulum movements are identical with segmenting contraction. Pressure recording classifications tell us nothing about the mechanical process involved and they are valued mostly as diagnostic tools in medicine.

Most important to our discussion is the fact that there is still disagreement as to the contraction sequence of circular and longitudinal muscle components in mammalian intestinal peristalsis (Bortoff and Ghalib, 1972; Gonella, 1972; Raiford and Mulinos, 1934; Bayliss and Starling, 1899; and Wood and Perkins, 1970). Obviously some standardization of terminology is necessary. The following, then, is an attempt to construct a useful definition of "peristalsis" consistent with its past and present usage.

Peristalsis is the phenomenon of any muscular contraction moving along a radially flexible tube in such a way that each component wave of circular, longitudinal or oblique muscular contraction is preceded and/or followed by a period of relative relaxation of all similarly oriented muscle within a given tubular segment. The meaning of this definition is not changed if the word "relaxation" is replaced by "contraction" and each "contraction" is replaced by "relaxation."

In any form of peristalsis, any two tubular segments are subject to a sequence of qualitatively identical force vectors. Furthermore, in locomotory peristalsis, each region of the body must, at some time during or between the passages of waves, have no forward motion. This is in contrast to the continuous undulatory locomotory mechanisms of fishes or the rectilinear progression of the boa constrictor (Gray, 1968), where the body moves as a unit and at a constant rate.

This definition is consistent with current and past use of the term "peristalsis" and includes the propagated waves of contraction found in vertebrate intestine, the retrograde locomotory waves found in earthworms and other coelomates, the locomotory waves characteristic of lepidopterous caterpillars (Barth, 1937), the direct locomotory waves of both dipteran larvae and *Euapta* and undoubtedly many more mechanisms of locomotion. Although the definition includes the locomotory waves of lepidopterous caterpillars, it excludes the sinusoidal locomotory waves of *Nereis* which involve alternating component waves of longitudinal contraction on opposite sides of the body cylinder (Gray, 1968). The definition also excludes swimming movements of leeches and fish, pedal locomotory waves, and the serpentine, concertine, crotaline and rectilinear movements of snakes (Gray, 1968). The peculiar swimming movements of young adult *Leptosynapta* are similarly excluded (Costello, 1946). The term, however, remains broadly applicable and an attempt to classify the types of peristalsis seems appropriate.

In the following classification of peristaltic types, all locomotory peristalsis will be classified as either direct or retrograde. The word "locomotory" is omitted to avoid verbosity. Only the names of non-locomotory waves will be without this direct versus retrograde distinction. Although some types of non-locomotory peristalsis may be mechanistically equated with forms of locomotory peristalsis, this distinction between locomotory and non-locomotory peristalsis is useful.

Transportive peristalsis as seen in the vertebrate intestine has not been fully characterized. Kosterlitz (1968), Raiford and Mulinas (1934), Wood and Perkins (1970) and Gonella (1972) believe longitudinal and circular muscle contract sequentially. Thomas and Baldwin (1971), Bortoff and Ghalib (1972) and Bayliss and Starling (1899) propose a simultaneous contraction of both circular and longitudinal muscle followed by a subsequent simultaneous relaxation.

A *Circular contraction peristaltic* wave is a wave of contraction of circular muscles only. *Euapta* has such a wave, and it cannot serve to pull the animal forward. It may serve some other purpose, as in the "ring of constriction" in *Thyone* (Pearse, 1908, page 266) which facilitates the detachment of tube feet from the substratum. The peristaltic wave described by Yamanouchi (1929) and Crozier (1915) may be circular contraction peristalsis. Yamanouchi's paper includes a diagram depicting a wave very much like the direct overlapping peristalsis of *Euapta* and he cites Crozier as having already described this type of wave in detail. Crozier, however, describes peristalsis in *Holothuria surinamensis* originating at any section of the body and slowly travelling either anteriorly or posteriorly. The body is not raised above the substratum and movement is accomplished primarily by the action of pedicles. Buccal tentacles and contraction of the body musculature play secondary roles in this locomotion.

Partially circular relaxation peristalsis consists of a wave of relaxation passing as an incomplete ring along the circular musculature. Coelomic fluid pressure causes distension at the point of relaxation. The worm *Arenicola* seems to use this type of wave to irrigate its' tubule although the role of longitudinal musculature is not delineated (Wells, 1961). In the case of *Arenicola* the wave of relaxation is restricted to the dorsal region. A wave occludes the space between the worm's dorsal surface and the walls of the tubule thus pushing water in front of it.

Longitudinal contraction peristalsis is a wave of contraction of only longitudinal muscles that is unrelated to locomotion. Sabella uses such a wave to irrigate its tubule (Mettam, 1969). Starting at one end of the body, a wave of longitudinal contraction shortens constant volume segments causing dilation. The dilated region closely fits the smooth tubule and the propagated wave acts like a "piston" traveling the length of the tubule and pushing water in front of it. Sabella's "piston" is able to move with minimal frictional resistance since parapodia are retracted and abundant mucous glands provide lubrication. Narrow elongated segments grip the tubule wall with extended parapodia.

Direct longitudinal peristalsis refers to waves of contraction of only longitudinal muscles travelling in the same direction as the animal. *Euapta* is capable of this wave type.

Direct arching peristalsis is observed in some lepidopterous caterpillars (Barth, 1937). Basically, a wave of contraction passes anteriorly along the dorsal longitudinal musculature and is followed by a wave of contraction of the dorso-ventral musculature. As the dorso-ventral musculature subsequently relaxes, a wave of contraction of the ventral musculature passes along the body tube.

Although they haven't been analyzed into their component muscular contractions, similarly described waves have been reported in holothurians. Parker (1921, page 205) reports a direct muscular wave in *Stichopus* which "resembles superficially the locomotion of a gigantic caterpillar." Glynn (1965, page 112) describes *Astichopus's* peristalsis in which "the posterior end is first elevated two to four centimeters from the substratum and then the wave moves forward forming an arch 2 cm high between ventrum and the underlying surface."

Direct overlapping peristalsis is the locomotory mechanism most frequently observed in *Euapta*. This type of peristalsis moves in the same direction as the animal. A wave of contraction of the longitudinal musculature passes along the body and during the latter part of its duration, occurs simultaneously with a wave of contraction of the circular muscles. Relaxation of circular and longitudinal muscles occurs simultaneously. The body cylinder's major axis is not elevated. In the burrowing polychaete *Polyphysia crassa* (Elder, 1973a) and in the apodous holothurian *Leptosynapta* (Elder, 1973b), the contractions of circular and longitudinal muscules appear to be simultaneous.

Direct overlapping partially circular peristalsis as demonstrated in *Euapta* is similar to direct overlapping peristalsis, differing only in the radial extent of circular contraction. In the former, contraction of circular musculature does not involve entire bands or rings of circular muscle as is the case for the latter.

Telescopic locomotion (Duncan and Pickwell, 1939), a *direct non-overlapping peristalsis* has been reported in some dipteran larvae. A direct wave of circular muscle contraction increases the pressure of the body fluids thus causing the body to lengthen and, pushing against a posterior fixed point, moves the head forward. While the head anchors itself to the substratum, a direct wave of contraction of longitudinal muscles then pulls the posterior regions forward. This appears similar to the burrowing activity observed in *Arenicola* (Truman, 1966).

Direct dilation peristalsis is reported in *Arenicola* (Mettam, 1969). In this type of locomotion (Wells, 1949) a direct wave simultaneously relaxes both circular and longitudinal musculature. Coelomic fluid pressure in the relaxed segment causes the body wall to dilate and contact the sides of the tubule, thus acting as a fixed point. Once the wave of relaxation passes, both circular and longitudinal muscle return to tonus. As the longitudinal muscle returns to tonus, longitudinal shortening pulls the tubular segment toward the fixed point which has in the meantime moved anteriorly.

Retrograde non-overlapping peristalsis is the method of locomotion of earthworms and is described in detail by Gray and Lissman (1938), Clark (1964) and Child (1901). Waves of contraction of longitudinal muscles pass posteriorly causing dilation of the body cylinder. A wave of contraction of circular muscles follows immediately, but does not overlap the longitudinal wave, and causes elongation of the body cylinder as well as causing it to push forward from more posterior fixed points. In some cases, as in earthworms, fixed points are provided by the increased body diameter. In some animals, fixed points are provided by other means of attachment. In the leech, for example, the suckers at each end of the body alternately serve as fixed points.

Retrograde dilation peristalsis is reported in Sabella (Mettam, 1969). A retrograde wave of contraction passes along the longitudinal muscle as the segmental volume is kept constant. Longitudinal shortening pulls a segment anteriorly toward a fixed point. Continued longitudinal shortening dilates the segment until it become wedged against the tubule walls and itself acts as a fixed point. After a wave of longitudinal contraction, a segment extends anteriorly.

These classes of peristalsis are not intended to be considered as the only possible classes. They do represent the different forms of muscular waves considered to be peristaltic that we have encountered in the literature.

Apodous holothurians comprise an unusual adaptation of hydrostatic skeletons.

Most animals that move by peristalsis have relatively thick body walls and high internal hydrostatic pressures. *Euapta* and other apodous holothurians are low pressure creatures whose body wall is so thin that it is ruptured by the weight of coelomic fluid if the animal is lifted into the air by one end. The Caribbean *Euapta* and the Pacific *Opheodesoma* live completely exposed on reef tops among corals and attached algae. They must normally be constant volume hydrostats—as are burrowing annelids. But these holothurians may, at any one time, contract the longitudinal muscles in the entire front (or rear) 2/3 of the animal and force the coelomic fluid into the remaining 1/3. This inflated 1/3 will have a larger diameter and no warts. Even those polychaetes that have incomplete septa cannot change their dimensions to this extent. As a result of their ability to change dimensions drastically, apodous holothurians can pass through cracks or holes in the substrate that are less than 1/3 their diameter. This recalls similar behavior of *Peripatus* (Manton, 1958) which is similarly correlated with lack of effective septa.

The structural dependence of holothurian warts upon hydrostatic pressure is reminiscent of the tube feet found in many other echinoderms. Yet while a retracted tube foot is still an anatomically identifiable structure, a flattened wart on *Euapta* is totally indistinguisable from adjacent regions of body wall.

We would like to thank Mrs. W. R. Heffernan for her selfless assistance in repeated typing and editing of the manuscript.

SUMMARY

1. The apodous holothurian, *Euapta lappa,* demonstrates four types of peristalsis. Direct overlapping peristalsis in which circular and longitudinal muscle components overlap is the most common. Other types are characterized by (a) being of longitudinal muscles only, (b) lack of circular muscle contraction along one side of the body and (c) being of circular muscles only (nonlocomotory).

2. "Warts" in apodous holothurians are not discrete structures but are areas of the body wall delimited by temporary contraction of circular muscles.

3. Peristalsis is defined as any muscular contraction moving along a radially flexible tube such that each component wave of circular, longitudinal or oblique muscle contraction is preceded or followed by a period of relative relaxation of all similarly oriented muscle. Definitions and nomenclature are given for 8 locomotory and 4 non-locomotory types of peristalsis.

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SOME FACTORS AFFECTING THE GROWTH AND DISTRIBUTION OF THE ALGAL ENDOSYMBIONTS OF *HYDRA VIRIDIS*

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Researches on the hydra-algae endosymbiosis over the last decade have produced a clearer understanding of the role of the symbiotic zoochlorellae. The works of Muscatine and Lenhoff (1965a, 1965b) have shown that green hydra survive and bud longer than aposymbiotic (algal-free) hydra under starvation conditions. Moreover, Muscatine (1965) and Muscatine and Lenhoff (1963) have demonstrated that the algae may release soluble, photosynthetically fixed material to the host's cells. Roffman and Lenhoff (1969) have shown that photosynthetically fixed $^{14}CO_2$ appears, with time, in all of the host's major biochemical fractions but predominates in the animal's glycogen pool. Analysis of the products released by the algae *in vitro* have shown them to consist mainly of the disaccharide, maltose (Muscatine, 1965). The assumption is that maltose is translocated to the host by the algae *in vivo* and serves as nutritional supplementation for the host during periods of starvation.

The ultrastructure of algae symbiotic with hydra has been studied by Park, Greenblatt, Mattern and Merril (1967) and Oshman (1967). These studies have shown that the algae are located in separate vacuoles within the digestive cells, and that the algae appear to be similar to free-living *Chlorella vulgaris,* a view advanced by Haffner (1925) and Beyernick (1890). Moreover the algae are seen to reside mainly in the base of the digestive cells (Whitney, 1907; Goetch, 1924; Haffner, 1925; Pardy and Muscatine, 1973).

Beyernick (1890) and Haffner (1925) claim to have cultured algae from green hydra, but repeated attempts by myself and others (Muscatine, personal communciation) to grow isolated hydra algae in pure culture have failed. As a result of the apparent inability to effect an axenic culture of the algal symbionts it is suggested that the symbiosis is obligate for the algae. Only recently has it been shown that the algae may derive nutrition from the host. Cook (1972) demonstrated that algae in hydra become radioactively labeled when the animals were fed ^{14}C labeled *Artemia* nauplii.

Current work has shown that aposymbiotic (algal-free) *Hydra viridis* are able to recognize potential algal endosymbionts and reject non-symbiotic algae (Pardy and Muscatine, 1973). These workers have shown that symbiotic algae injected into the gastrovascular cavity of aposymbiotic hydra are phagocytosed and transported to the base of the animals' digestive cells. With time the injected algae reestablish the symbiosis and restore the standing population of endosymbionts.

Virtually nothing is known as to how the endocellular algal flora may be regulated or modulated by the host. Unstudied is the effect of various environmental conditions on the size and distribution of the resident algal flora. This paper describes the pattern of algal distribution in the hydra, how this pattern may be altered, and how continuous light, dark, and the animal's nutrition may affect the size of the algal population maintained by hydra.

MATERIALS AND METHODS

Maintenance of hydra cultures

Stock cultures of *Hydra viridis* (Florida strain) were maintained in M solution (Muscatine and Lenhoff, 1965a) at room temperature and room illumination (approximately 50 foot-candles), hereafter referred to as ambient conditions. The animals were fed daily to repletion on freshly hatched *Artemia* nauplii. For dark experiments, animals were placed in 15 cm plastic petri dishes which were then wrapped with aluminum foil. These animals were exposed to no more than 2–4 minutes of light daily for counting, feeding and routine maintenance. In experiments requiring animals to be exposed to continuous illumination, animals in plastic petri dishes were placed on a white background 25 cm below two 20-watt Sylvania Gro-Lux lamps.

Technique for counting the number of algae in hydra

Endocellular algae were counted in digestive cells that had been isolated from hydra by maceration (David, 1973). In practice, either an individual or a piece of hydra was transferred to a drop of maceration fluid on a microscope slide. This solution consisted of glacial acetic acid, glycerine and water (1 : 1 : 13-v : v). After 5 minutes in this fluid the individual cells were teased apart with fine dissecting needles. Figure 1 is an example of a digestive cell prepared in this manner and demonstrates the presence of endocellular algae. The average number of algae/digestive cell was determined by counting directly the algae in digestive cells prepared by maceration.

In an exponentially growing population of hydra there are individuals in all states of development, of varying size, and possessing a variable number of budding hydranths. Thus the selection of a "standard" experimental animal is somewhat arbitrary. For the present work a standard hydra was defined as an animal that had two budding hydranths in an advanced state of growth.

To estimate the total number of algae in hydra, 10 standard animals were homogenized in 1 ml of M solution using a semi-micro tissue homogenizer. An aliquot of homogenate was transferred to a hemacytometer where the number of algae in the sample was determined. Counts from 5 groups of hydra were averaged and extrapolated to yield the average number of algae per hydra. In some experiments it was necessary to determine the standing crop of algae in an entire growing population of hydra. Since growing populations of hydra have individuals in various stages of development as mentioned earlier, a more useful index was found to be the number of algae per *hydranth*. Therefore the number of algae counted from homogenates of whole populations was expressed as the number of algae per hydranth.

Inspection of intact hydra reveals that the animals are not uniformally green. The central region of the hydra appear greener than the stalk and base. Such an observation has been reported previously by Pardy and Muscatine (1973). As various environments and physiological factors might influence the distribution of algae within the animals, it was important to assess quantitatively the apparent heterogenous distribution of algal symbionts. To do this animals were divided into three zones (Pardy and Muscatine, 1973): Zone 1, hypostome and tentacles; Zone 2, central growing region; Zone 3, stalk and base. Zones were dissected from ten individual hydra (buds removed) and macerated separately. The number of algae in 20 digestive cells from each zone was counted. In experiments dealing with the effects of light and feeding on the number of algae per digestive cell (discussed below), only cells from Zone 2 were analyzed.

Effect of light, dark and feeding on the number of endocellular algae

To determine the effect of light and nutrition on the number of endocellular algae, cultures of hydra were maintained under the following conditions: (1) ambient light conditions—fed every 24 hours, (2) ambient light conditions—starved, (3) constant illumination—fed every 24 hours, (4) constant illumination—starved, (5) constant darkness—fed every 24 hours, (6) constant darkness—starved.

Animals for these experiments came initially from populations in exponential growth maintained under ambient light conditions and fed every 24 hours. Hydra from such cultures had 18 ± 3.1 (Zone 2) algae per digestive cell (n = 100). At 24-hour intervals 100 digestive cells (20 from each of 5 animals were selected from each of the experimental groups—Zone 2) were examined and the number of algae in each cell was recorded. The data were expressed as the mean number of algae ± s.d. per digestive cell.

Growth rate of hydra

Hydra's growth rate was measured according to the method of Loomis (1954). Standard animals with two buds were placed in 8 cm plastic petri dishes. Every morning the total number of hydranths was counted, the number recorded, and the animals fed to repletion. The average number of hydranths from duplicate experiments was plotted on semi-log paper against time. From a straight line fitted to these points, K, the growth rate constant was calculated using the standard equations for exponential growth.

Growth rate of endosymbiotic algae

Ten dishes each containing 20 hydranths were maintained in either continuous illumination or constant dark and fed every 24 hours. At 24-hour intervals following the start of the experiment for a total of 5 days, the total hydra population in a dish was harvested and homogenized in 1 ml of M solution. The number of algae in an aliquot of the homogenate was determined by using a hemacytometer as described earlier and the average number of algae from two dishes was plotted on semi-log paper against time. Growth rates of the algae were calculated in the same manner as those for hydra.

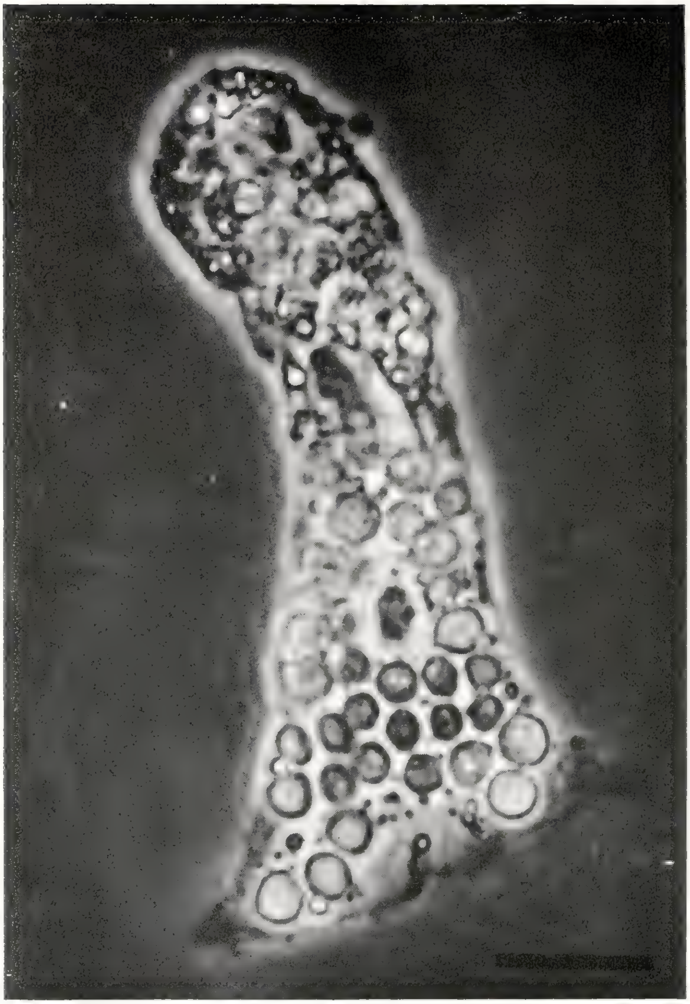

Figure 1. A digestive cell from green hydra prepared by maceration and photographed with phase optics. Symbiotic algae can be seen in the base of the cell. The animal cell nucleus with its prominent nucleolus is located centrally. Bar equals 10 microns.

Results

Examination of cells from hydra prepared by maceration (Fig. 1) showed that the algal symbionts resided mainly in the base of the digestive cells. All digestive cells examined contained algae though individual digestive cells evidenced considerable range (2–30) in the number of algae contained in them. I found approximately $1.5 \times 10^5 \pm 3.7 \times 10^4$ algae per hydra in a standard intact *Hydra viridis,* a value closely agreeing with that of Pardy and Muscatine (1973).

Figure 2 is a histogram showing the distribution of algae in the three zones of hydra. It is evident that the algae are not uniformly distributed throughout the animal. Zone 2, the central growing region, averaged approximately 19 algae per digestive cell, whereas Zones 1 and 3 had fewer algae cells, averaging about 12 symbionts per digestive cell.

Animals grown in continuous darkness became increasingly pale, however, I observed that Zone 3 appeared to be greener than Zones 1 and 2. As this condition was different from animals maintained in continuous light (see above), I

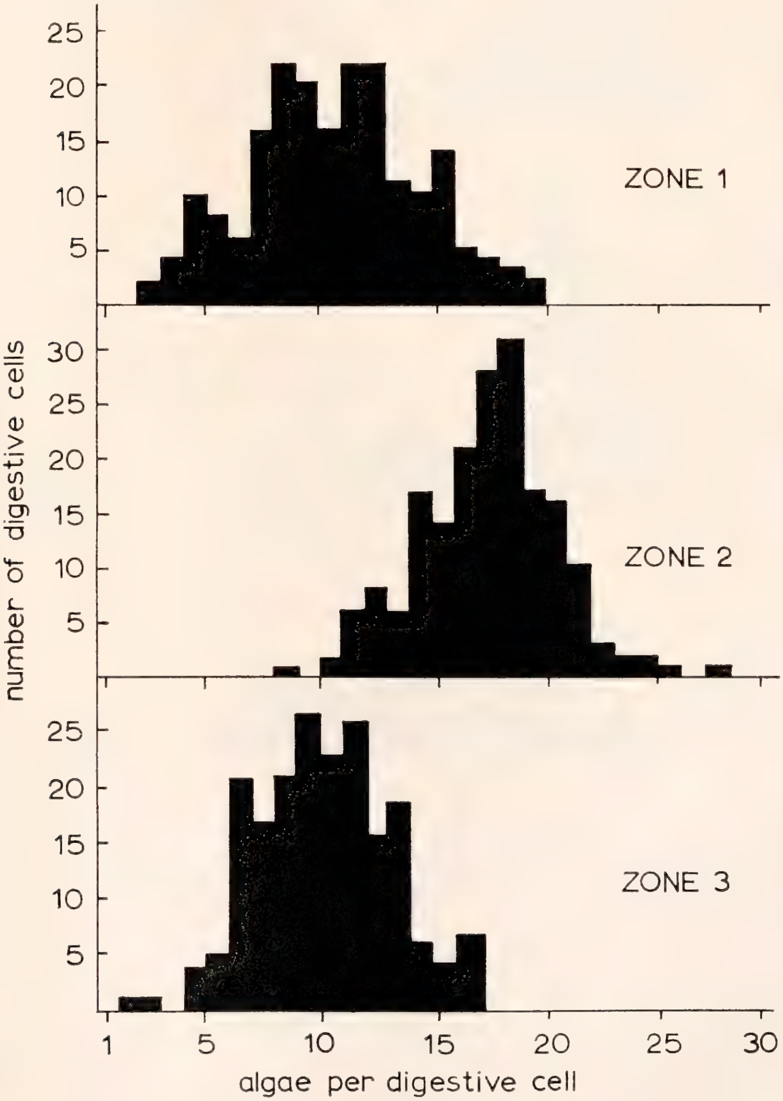

FIGURE 2. Histogram showing the distribution of algae in three zones of green hydra maintained in constant illumination and fed daily; Zone 1—tentacles and hypostome, Zone 2—central growth region, Zone 3—stalk and basal disc.

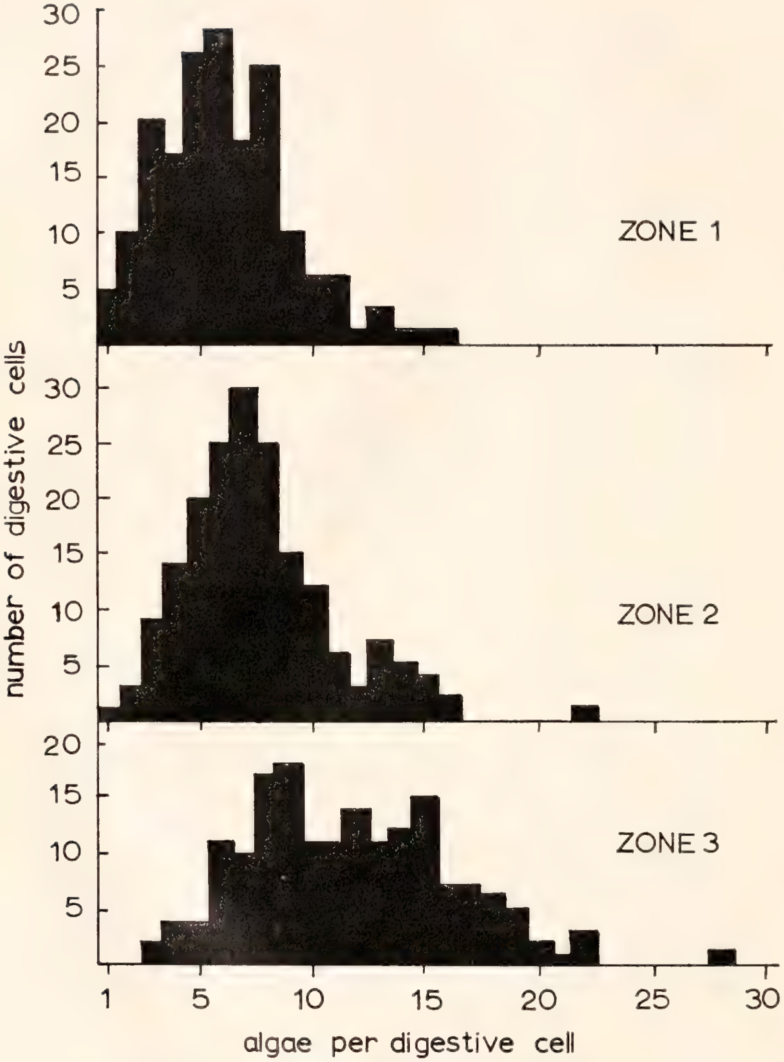

FIGURE 3. Histogram showing the distribution of algae in three zones of green hydra maintained in continuous dark for 6 days. Zones are the same as Figure 2.

examined the distribution of algae in the three zones from hydra grown in the dark. Figure 3 is a histogram of the distribution of algae from hydra grown in the absence of light for 16 days. Compared to the zones from hydra reared in continuous light (Fig. 2), the results shown in Figure 3 may be summarized as follows: Zones 1 and 2 have fewer algae (averaging approximately 6 and 7 per digestive cell, respectively). Zone 3 had approximately the same number of algae (11 symbionts per digestive cell) as hydra grown in continuous light. These data show that the numbers and proportions of algae in Zones 1 and 2 (Fig. 2) are not fixed but may be altered by growing the animals in continuous darkness (Fig. 3). During growth in the dark, the number of algae are reduced in Zones 1 and 2, but apparently not in Zone 3.

Table I shows the effects of the various illumination and feeding conditions on the number of algae in digestive cells from hydra. , Hydra which are fed and maintained under ambient conditions exhibited a nearly constant number of algae per digestive cell over the duration of the experiment. The remainder of the experimental results can be grouped in two classes: those in which there is a small, transient increase in algae followed by an approach to starting levels (Table I, ambient-starved, constant light-fed, constant light-starved), and those in which a pronounced, continuous decrease in the number of algae per digestive cell was observed (Table I, constant dark-fed, constant dark-starved).

TABLE I

Number of algae/digestive cell (Zone 2) from animals maintained under various conditions of light and feeding (see Materials and Methods). Data are expressed as mean ± s.d. of 100 digestive cells (20 from each of 5 hydra). Animals for the experiments were selected from a population of hydra that averaged 18 ± 3.1 algae/digestive cell (Zone 2) on day 0

Experimental condition	Day				
	1	2	3	4	5
Ambient-fed	18.2 ± 2.8	17.0 ± 2.0	17.0 ± 3.1	18.4 ± 2.6	17.9 ± 3.2
Ambient-starved	20.4 ± 3.1	20.2 ± 4.1	20.1 ± 3.6	18.3 ± 2.8	18.0 ± 3.0
Constant light-fed	18.1 ± 4.6	20.5 ± 4.7	19.6 ± 3.8	20.8 ± 4.1	19.1 ± 4.5
Constant light-starved	20.0 ± 4.1	21.0 ± 5.7	22.2 ± 3.8	21.8 ± 4.6	18.3 ± 3.3
Constant dark-fed	13.5 ± 3.8	12.3 ± 3.8	10.6 ± 3.7	6.8 ± 2.3	6.6 ± 3.1
Constant dark-starved	17.6 ± 3.8	17.0 ± 1.4	12.5 ± 3.4	11.0 ± 3.0	11.0 ± 3.2

Figure 4 is a semi-log plot of the increase in the number of algae and hydra in cultures maintained under continuous illumination and fed to repletion every 24 hours over a period of 4 days. The growth rate constant of hydra under these conditions was $K = 0.358$ and of the algae, $K = 0.380$. The doubling time for the hydra and algae was approximately 1.9 days.

The result of growing hydra under conditions of continuous dark while feeding every 24 hours is shown in Figure 5. It is evident that the hydra population keeps expanding exponentially though at a rate ($K = 0.288$) somewhat less than hydra maintained in continuous light (Fig. 2). The algae undergo only a very slight increase ($K = 0.026$) compared with those in the continuous light experiment ($K = 0.380$).

When the average number of algae per hydranth from populations of hydra maintained under continuous light and dark is plotted against time on semi-log coordinates, the curves shown in Figure 6 are obtained. From these graphs it is apparent that the average number of algae per hydranth remains nearly constant in populations grown under continuous illumination. Conversely, the number of algae per hydranth in hydra grown in continuous dark declines ($K = -0.232$).

Inspection of Figures 5 and 6 suggests that the decrease of algae observed in hydra which are grown in the dark may be correlated with the exponential growth of the animals. If the rate of algal decrease ($K = -0.232$) is corrected for the slight rate of algal division actually observed in the dark ($K = 0.026$) (Fig. 5), a new rate, $K = -0.258$ is obtained. This value represents the theoretical rate of algal decline to be expected in dark-grown hydra if the algae were to cease dividing. Hence the rate of algal decrease should equal the growth rate of the animal hosts. The theoretical rate of algal decreases given above ($K = 0.258$) approximates the rate of animal multiplication ($K = 0.288$) and indicates that the loss of algae from hydra reared in the dark probably results from dilution of the standing crop of algae brought about by repeated animal cell division.

The results from the experiment depicted in Table I (constant dark-fed), and Figures 4, 5 and 6 clearly emphasize the role of light in maintaining continuous maximum algal growth. Thus an experiment was devised to determine how long it would take the algae to repopulate hydra following depletion resulting from exponential-growth of the animals in the dark. Duplicate cultures of hydra were maintained in continuous darkness and fed every 24 hours. After 11 days it was determined that the hydra had approximately 7.1×10^3 algae per hydranth. The cultures were then placed in continuous light and the average number of algae per hydranth was determined every 24 hours for 4 days. Figure 7 shows that there

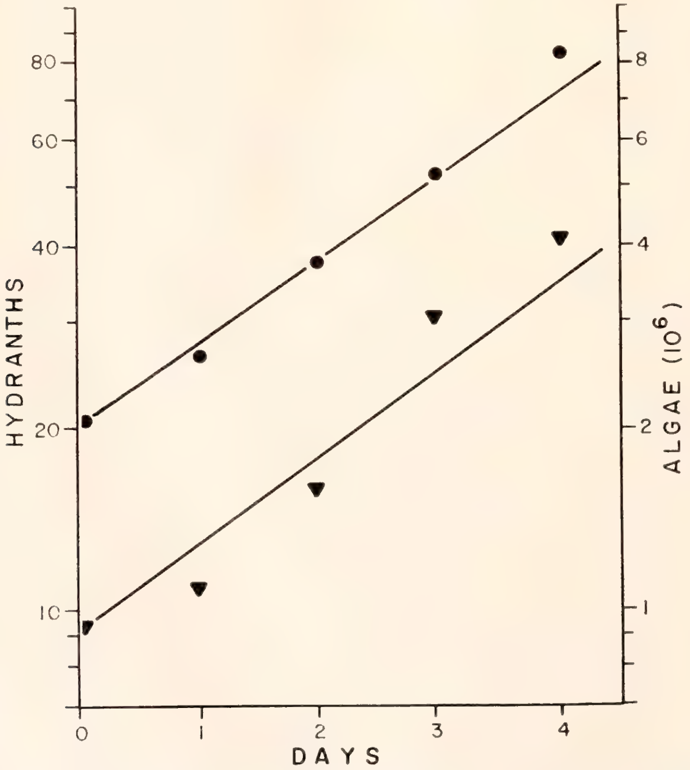

FIGURE 4. Growth of hydra and algae under continuous illumination and daily feeding—circles, hydranths; triangles, algae; growth rate of hydra, $K = 0.388$; growth rate of algae, $K = 0.380$.

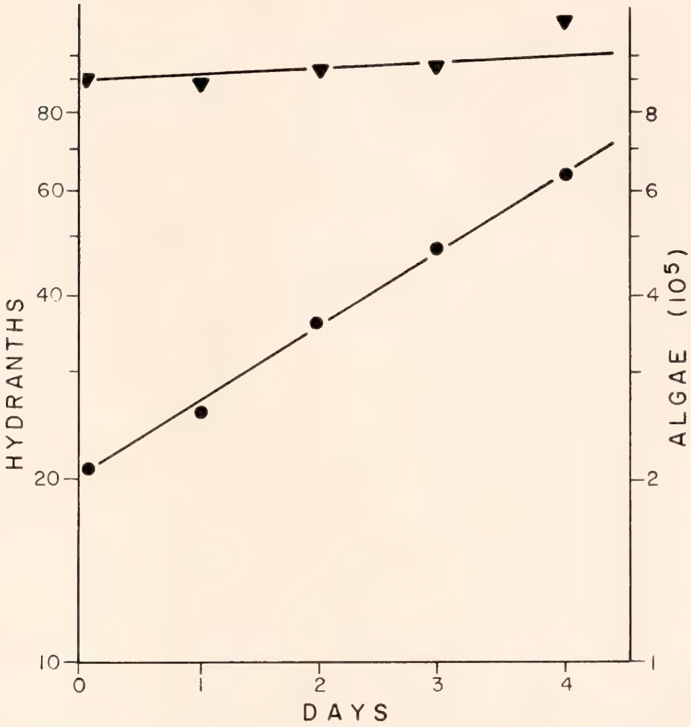

FIGURE 5. Growth of hydra and algae in continuous darkness and with daily feeding—circles, hydranths; triangles, algae; growth rate of hydra, K = 0.288; growth rate of algae, K = 0.026.

was a burst of algal multiplication over the first 2 days following the return of the animals to light. During this initial period the algae exhibited a growth rate of 1.37—over three times the typical algal rate. After the period of rapid multiplication the algal population reached and maintained a level typical of hydra grown in continuous light.

DISCUSSION

The effect of light and feeding on the growth of algae in hydra can be viewed as the consequence of two distinct but interrelated factors: the necessity of light for maximum algal multiplication, and tissue growth in hydra. Light has been shown to be a necessary condition for a variety of algal processes (photosynthesis, organic and inorganic nutrient assimilation, ion uptake) including division in certain strains of *Chlorella vulgaris* (Griffith, 1961). Algal multiplication in green hydra is strongly light dependent. Figure 5 shows that when hydra are transferred to the dark the algal population increased only slightly (K = 0.026). Under constant light and feeding, the number of hydra increases at an exponential rate of K = 0.358 and the algae increase at approximately the same rate (K = 0.380) (Fig. 4). These results are expected if animals in a growing population of hydra are to maintain a constant, optimum number of algae, and Table I,

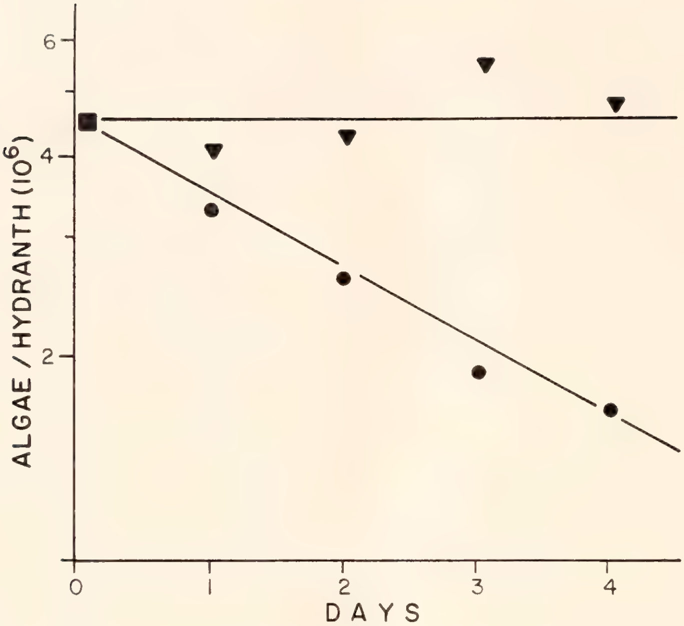

FIGURE 6. The number of algae per hydra in continuous light compared to the number of algae per hydra in animals maintained in continuous darkness. Both groups fed daily; the rate of decrease of algae, K = −0.232; triangles, continuous light; circles, continuous dark.

ambient-fed constant light-fed, and Figure 4 indicate that the hydra do maintain a relatively constant number of algae during exponential growth in the light.

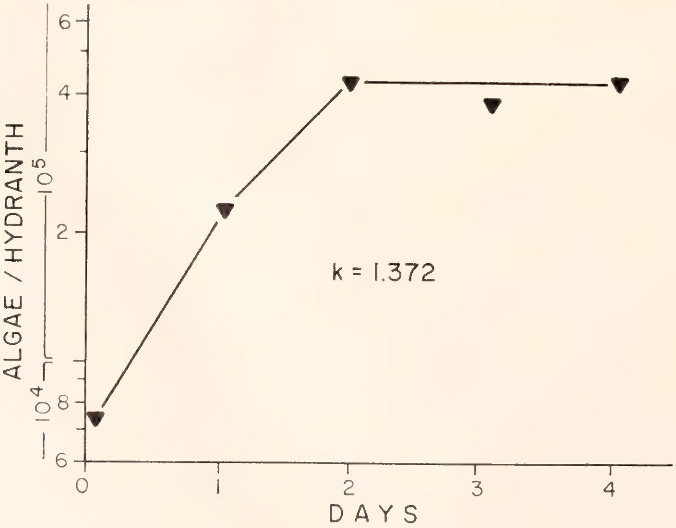

FIGURE 7. Growth of algae in hydra after being transferred to continuous light following 11 days in constant darkness; animals fed daily.

When hydra is reared in the absence of light, the number of algae per digestive cell (Zone 2) and per hydranth rapidly declines (Table I, constant dark-fed; Fig. 6). The algae continue to grow in the dark though very slowly (Fig. 5). The depopulation of algal symbionts observed in animals reared in the dark results from the growth and asexual reproduction of the host. The animals keep proliferating at a high rate relative to the algae and hence outgrow their symbionts.

Further support for this argument comes from Table I, constant dark-starved, which shows that when hydra are starved in the dark fewer algae are lost from the digestive cells than when hydra are fed (Table I, constant dark-fed). In starved hydra the rate of animal growth decreases (Muscatine, 1961) and with time the hydra cease multiplying altogether. In the absence of hydra growth in the dark, the apparent loss of algae also stops. I have kept starved animals in the dark for up to 10 days without observing any loss of green color.

It could be postulated that exponentially growing hydra maintained in the dark would eventually outgrow their algal symbionts. In fact, such an effect has not been observed. I have kept growing cultures of hydra in the dark for as long as four months with the animals maintaining a low level of infection that reproduces at the same rate as the hydra. It is possible that in the dark the algae assume a heterotrophic mode of nutrition and derive all of their energy by the assimilation of metabolites from the hydra. The total number of heterotrophic symbionts that are maintained under dark conditions would thus be determined by density-dependent factors such as competition for nutrition available from hydra. Cook (1972) has shown that transfer of material from hydra to algae takes place. Hydra kept in both light and dark were fed ^{14}C labeled *Artemia* nauplii. Assay of the algae after 48 hours showed that they had acquired 25–43% of the total radioactivity in the dark *versus* 22–26% in the light. While the nature of the translocated substance(s) is not known, Cook's work (1972) clearly demonstrates that a flow of material from hydra to the algae takes place. This material is probably the source of heterotrophic nutrition for the algae.

There is evidence suggesting that algae in the dark may be competing with hydra for metabolites. I have found that green hydra grows slower in the dark (Fig. 5, $K = 0.288$) than in the light (Fig. 4, $K = 0.358$) and that the animals growth rate may be depressed by as much as 20% in the dark. Thus in the dark there may be two levels of competition—between algae and between algae and host—the sum of which has the overall effect of depressing the growth of both symbiotic partners when they are in the dark. The absence of light, however, has an overall greater effect on the growth of the algae.

My data show that hydra reared in the light have most of their endosymbionts located in the central growing region (Fig. 2). This situation may reflect that some essential nutrient(s) supplied by the hydra cells of the growth region and needed for maximal algal growth are limiting in the hypostomal (Zone 1) and basal (Zone 3) regions. Alternatively, hydra may actively regulate algal mitosis via some hormone-like mechanism, promoting division in the growth region and/or suppressing it in the basal and hypostomal region. Finally, algal mitosis might in some way be influenced by digestive cell mitosis. While there is no evidence to support these ideas, the work of Campbell (1967) is suggestive. Campbell (1967) demonstrated that while mitosis occurs throughout the body of the animal, the greatest amount of mitotic activity occurs in those cells (digestive, epithelial-muscular) of the central growing region with a substantially lower level in the base and hypostomal areas (Campbell, 1967). Recently, David and Campbell (1972) have found that the cell cycle of epithelial cells in the basal disk and peduncle of *Hydra attenuata* is longer than those in the growing regions. The zonal distribution of algae that I have found in green hydra appears to correspond to the pattern of actively mitosing cells found in other hydra. Possibly the processes which initiate or are involved in mitosis of the hydra cells promote algal division.

Hydra maintained in the dark exhibit a pattern of algal distribution (Fig. 2) differing from animals reared in the light (Fig. 3). In dark-reared animals there are more algae in the base (Zone 3) than in the other zones. Such a situation may be interpreted as follows: Loss of algae from hydra reared in the dark is a result of dilution of the standing crop of algae by the division of the digestive cells and is proportional to the growth rate of hydra. Because the animal cells constituting the various zones probably do not divide at the same rate (Campbell, 1967), the differential loss of algae observed in the various zones may reflect the differential mitotic activity of the digestive cells in these regions. The digestive cells of Zone 3 divide slower than the cells in the other zones and hence, in the dark, have proportionally more algae.

Though direct experimental evidence is lacking, it is generally agreed that the endosymbiotic algal population in hydra is under some kind of regulation. The algae do not over-grow their hosts and seem to maintain a fairly constant number providing there is adequate light (Table I, ambient-fed, ambient-starved, constant light-fed). Possibly hydra actively regulates the division of its algal flora, maintaining a population compatible with the physiology and metabolism of the digestive cells. Alternatively, the ultimate number of algae in digestive cells (in the presence of light) may be density dependent. The quantity of algae could be limited either by competition among fellow symbionts for growth-promoting substances supplied by the hydra or by growth-inhibiting factors released by the algae themselves. Hydra transferred to constant light, whether fed or starved, show a transient increase in the number of algae per digestive cell (Table I, constant light-fed, constant light-starved). Animals starved under ambient conditions also show this effect. If the algae are regulated by hydra, the slight algal increase observed may represent a temporary disruption in the control of algal division by the host or by a response to a change in physiological conditions, *e.g.*, feeding and light.

Hydra depleted of their algae as a result of rearing them in the dark experience a rapid repopulation by their symbionts when the animals are returned to the light (Fig. 7). Under these conditions the algae multiply at a rate nearly four times greater than under normal conditions. Rapid growth of the algae continues until the population reaches normal levels. Fast growth followed by plateauing under the conditions just discussed could be interpreted as either a result of density-dependent phenomena, active regulation by the hydra, or a combination of these processes. That light apparently triggers fast growth following prolonged maintenance in the dark is consistant with the behavior of other *Chlorella*. Griffith (1961) reported that cells of *Chlorella vulgaris* maintained in the dark accumulate in a predivision phase. Such cells undergo rapid, multiple division when placed in the light.

I would like to dedicate this work to the memory of Dr. Berton Roffman, whose promising career was ended by his tragic and untimely death in 1972. Dr. Roffman had just begun an exhaustive biochemical study of the hydra-algae symbioses.

Summary

1. Under conditions of continuous illumination and daily feeding, individual specimens of *Hydra viridis* possess approximately 1.5×10^5 endosymbiotic algae.

2. The endosymbiotic algae are found in greatest abundance in those digestive cells constituting the central growth region (Zone 2) of the hydra ($\approx$ 19 algae/digestive cell) with fewer algal cells ($\approx$ 12 algae/digestive cell) residing in the hypostome, tentacles (Zone 1) and stalk and basal disc (Zone 3).

3. Under steady-state growth in the light, the algae reproduce at approximately the same rate as the animal hosts—$K = 0.380$ and 0.385, respectively.

4. Endosymbiotic algae require light for maximum reproduction and green hydra grown in the dark show a decrease in the number of their algal symbionts. The rate of algal decline in the dark is believed to result from a dilution of the standing crop by the continuous growth of the animal tissues. Support for this theory is based on the observation that the rate of algal decline in the dark ($K = 0.258$) approximates the rate of animal growth ($K = 0.288$).

5. The algae in hydra maintained in the dark exhibit a different pattern of distribution in the host than those in the light. Apparently loss of symbionts in the dark takes place differentially with proportionately more algae being lost from the tentacles, hypostome and growth region than from the stalk and basal disc.

6. When hydra that have been maintained in the dark are returned to the light, their endosymbiotic algae undergo a rapid multiplication and repopulate the host in approximately two days.

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JUVENILE HORMONE AND THE ADULT DEVELOPMENT OF *DROSOPHILA*

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It is clear that hormones control insect metamorphosis (see for example Wigglesworth, 1970; Wyatt, 1972; Doane, 1973), and that gene activity is involved (Ashburner, 1972a; Shearn, Rice, Garen and Gehring, 1971; Stewart, Murphy and Fristrom, 1972), but the precise interaction of the hormones with the genome is little understood. Williams and Kafatos (1971) have proposed a model involving juvenile hormone-controlled gene switching. Because of the hormone's involvement in genetic mechanisms, there is an increasing need for investigation of insect species which can be manipulated both genetically and cytogenetically. Genetic variations in *Drosophila* which alter endocrine functions have already been described. Juvenile hormone metabolism is altered in adults of a female sterile mutant (Postlethwait and Weiser, 1973), and several mutants affect ecdysone levels in mature larvae (Rayle, 1967; Karlson and Hanser, 1952).

Developmental aberrations have been described for *Drosophila* after treatment with juvenile hormone (JH) or certain JH analogs (Dearden, 1964; Bryant and Sang, 1968, 1969; Ashburner, 1970b; Madhavan, 1973). In these studies JH caused bristle modifications, failure of genitalia to rotate, and inhibition of eclosion. Interpretation of these effects on *Drosophila* has been complicated because there has as yet been no comparison of the abnormalities induced by exogenous application of JH analogues and the effects of juvenile endocrine organs implanted into ready-to-pupate hosts. This comparison is the first point studied in the present report: The developmental aberrations produced by the application of JH are compared to those provoked by the implantation of larval fragments containing living, active brain-ring gland complexes, the source of JH in the higher Diptera (Vogt, 1943, 1946).

If the genetics of *Drosophila* is to be exploited to help unravel the genetic basis of the hormonal control of insect development it would be best if a Dipteran juvenile hormone could be employed in such studies. But, if a Dipteran JH is to be characterized, a convenient and sensitive assay must be available. The second point approached by this communication is a JH assay for *Drosophila* which can detect as little as 2 picograms of Cecropia JH. The sensitive developmental period is defined, and several JH analogues are ranked with regard to their JH activity in *Drosophila* to provide information on the parts of the molecule important for biological activity.

MATERIALS AND METHODS

Transplants

Drosophila virilis and *Drosophila melanogaster*, Oregon R wild strain, were cultured at 25° C using standard procedures. In order to test the effect of juvenile endocrine organs upon mature *Drosophila* larvae, first instar larvae 2–5 hours after hatching were cut into thirds in *Drosophila* Ringers (Chan and Gehring, 1971), and the anterior or posterior third was transplanted into a mature third instar host of the same species (see Ursprung, 1967, for transplantation techniques). Larval hosts were those which had ceased feeding and had crawled up onto the wall of the culture bottle to pupariate. In other experiments first instar brain-ring gland complexes, first instar mouth hooks, or late third instar imaginal discs were implanted into mature larval hosts. After metamorphosis the host was dissected and the position of the implant recorded. The cuticle of the host and the implant were then mounted in Gurr's water mounting medium between two cover glasses, and examined under 800 × magnification.

Staging for hormone treatments

For hormone applications developmental stage was reckoned with respect to pupariation—the time at which the larva becomes immobile, everts its anterior spiracles, and forms the puparium. The puparium remains white for only about a half hour (commonly called "white pre-pupa" stage); so this phase provides a convenient and accurate standard developmental stage. For experiments testing hormone sensitivity prior to pupariation, late third instar larvae were collected, anesthetized with ether, and treated with hormone or control solutions. Pupariated animals were collected hourly thereafter. For experiments testing hormone sensitivity after pupariation, white prepupae were collected and treated with hormone at appropriate times thereafter. A few pupae were etherized prior to acetone or hormone treatment. These animals gave results similar to those obtained without etherization; so in general, neither control nor experimental pupae were etherized.

Normal animals emerge from their puparia as adults four or five days after pupariation. Experimental animals were fixed in 70% ethanol seven days after pupariation, whether they had eclosed or not.

Application of solutions

Hormones were dissolved in Mallencrodt nanograde acetone. Controls were treated with acetone alone. Solutions were dispensed topically onto the dorsal posterior surface of the animals, except where noted in the text. Solutions were dispensed through a micro-pipette with a tip approximately 0.2 mm in diameter. A 100 μl Hamilton syringe was driven by a syringe pump for a calibrated period of time to deliver a convenient volume. Calibration showed that about 0.3 μl was delivered per dose.

Hormones

The following chemicals were tested according to the procedure described above: I., methyl 3,11-dimethyl-7-ethyl-*cis*-10,11-oxido-*trans,trans*-2,6-tridecadienoate (C18-JH); II., methyl 3,11-dimthyl-7-ethyl-*cis*-10,11-imino-*trans,trans*-2,6-tridecadienoate (C18-JH-aziridine); III., methyl 3,11-dimethyl-7-ethyl-*trans,trans, cis*-2,6,10-tridecadienoate (Methyl bishomofarnesoate); IV., 3,7,11-trimethyl-*cis, trans,trans*-2,6,10-dodecatrienoate (Farnesenic acid); V., 3,7,11-trimethyl-*cis,trans, trans*-2,6,10-dodecatrienol (farnesol); VI., methyl 3,7,11-trimethyl-*cis*-10,11-oxido-

trans,trans-2,6-tridecadienoate (C17-JH); VII., methyl 3,7,11-trimethyl-*cis*-10,11-oxido-*trans,trans*-2,6-tridecadienaldehyde (C17-JH aldehyde); VIII., methyl 3,11-dimethyl-7-ethyl-10,11-oxido-*trans,trans*-2,6-dodecadienoate (iso-C17-JH); IX., methyl 3,7,11-trimethyl-10,11-oxido-*trans,trans*-2,6-dodecadienoate (C16-JH); X., methyl 3,711-trimethyl-10,11-imino-*trans,trans*-2,6-dodecadienoate (C16-JH aziridine); XI., methyl 3,7,11-trimethyl-10,11-thio-*trans,trans*-2,6-dodecadienoate (C16-JH-episulfide); XII., methyl 7-chloromethyl-10,11-oxido-3 methyl-*trans,trans*-2,6-dodecadienoate (7 chloromethyl C16-JH); XIII., 3,7-dimethyl-6,7-oxido-*trans*-2-octenyl-3′,4′-methylene-dioxyphenyl ether (epoxygeranyl sesamole); XIV., 3,7-dimethyl-*trans*-2,6-octenyl-3′,4′-methylenedioxyphenyl ether (geranyl sesamole); XV., 3,7-dimethyl-6,7-oxido-*trans*-2-octenyl-4′-ethylphenyl ether (Stauffer R-20458); XVI., 3,7-dimethyl-6,7-oxido-*trans*-2-octenyl-4′-isopropylphenyl ether (Stauffer R-19828); XVII., methyl 3,7-dimethyl-6,7-oxido-*trans*-2-octenyl-4′-benzoate; XVIII., methyl 3,7-dimethyl-*trans*-2,6-octenyl-4′-benzoate; XIX., ethyl 7,11-dichloro-3,7,11-trimethyl-*trans*-2-dodecenoate (ethyl dichlorofarnesoate); XX., Law-Williams mixture (Law, Yuan and Williams, 1966); XXI., Sesamex; XXII., isopropyl 11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate, technical grade, (Zoecon ZR-515).

The author is grateful to Dr. Alfred Ajami for providing III, IV, VII-XI, XII-XX, (see Ajami, 1973, for procedures of synthesis and purification), to Professor E. J. Corey for providing I, II, VI and XII, and to Zoecon Corporation for XXII.

Data collection

The rotation of male genitalia was estimated in the stereo-microscope before dissection. The angle between the dorso-ventral axis of the fly and the axis of symmetry of the genitalia was estimated to the nearest 30°. Since the genitalia rotate clockwise during normal development, angles were estimated from 0–360° in a clockwise direction.

After measuring genitalia rotation, abdomens were removed from the animals, and internal structures dissected away. All cuticular structures including the head and thorax were then carefully mounted between two coverslips in Gurr's water mounting medium to check for hormonal effects at a magnification of approximately 800 ×. The surface architecture of the abdominal cuticles of a number of experimental and control *D. melanogaster* was studied in detail by scanning electron microscopy. Freshly prepared abdomens were examined after coating them *in vacuo* with carbon and gold-palladium.

The standard assay was 15 to 20 individuals per point in Figure 4. For the dose-response curves, each point represents two to four standard assays. The same individuals were used to study all four parameters of Figure 4, and where appropriate, in Figure 6. A total of 1071 C18JH-treated animals and 208 acetone treated controls provide the data for Figures 5 and 7. An additional set of 381 animals provide the data in Table III (the analogue assays).

RESULTS

Effect of juvenile endocrine organs on the metamorphosis of Drosophila

The first series of experiments were designed to find the effect of juvenile endocrine organs upon the epidermis of a metamorphosing host. *D. virilis* and *D.*

TABLE I

The effect of implanted parts of first instar larvae upon metamorphosing Drosophila virilis *hosts*

Implant	Age of implant	Number of recovered hosts	Number of hosts with aberrant cuticle above implant
Anterior third	First instar	84	17 (20%)
Posterior third	First instar	87	1 (1%)
Anterior third minus brain-ring gland complex	First instar	99	5 (5%)
Brain-ring gland complex	First instar	74	14 (19%)
Imaginal disc	Third instar	48	0 (0%)

melanogaster were used as donors and hosts. The data for both were similar, but were more extensive for *D. virilis,* and so these appear in Table I.

When abdomens of adult hosts were examined under the compound microscope, abnormalities were evident in 17 (20 per cent) of the 84 abdomens that had been implanted with the anterior third of first instar donors. Only 1 abnormality was encountered among 87 abdomens that had received the posterior third of first instar larvae. The abnormalities were localized in the cuticle immediately overlying the implant; they were encountered only when the implant was superficial, never when it was deeply imbedded in the abdomen. As illustrated in Figure 1, the

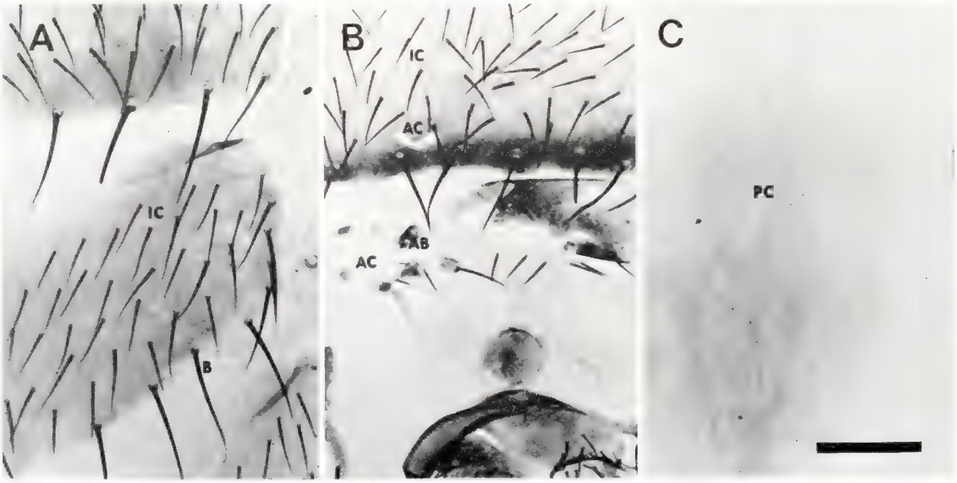

FIGURE 1. The effect of implanted juvenile endocrine organs on a metamorphosing host; A., Control adult abdominal cuticle from a host implanted with the posterior third of a first instar larva; B., Adult abdominal cuticle from a host implanted with the anterior third of a first instar larva; C., Normal pupal cuticle. Symbols used are AB., abnormal bristles; AC., aberrant cuticle; B., normal bristles; IC., imaginal cuticle; PC., pupal cuticle. Scale bar equals 100 μ.

abnormalities consisted of a localized zone closely resembling pupal cuticle and differing from adult cuticle in terms of the absence of pigment and hairs. So also, the bristles distinctive of adult cuticle were either absent or of aberrant size and shape.

Vogt (1946) apparently observed similar inhibition of adult differentiation after the implantation of *adult* corpora allata into maturing third instar hosts of *D. hydei*.

The ring gland of first instar larvae proved to be too small to be dissected and transplanted as such. Therefore, the brain-ring gland complex was removed and implanted into mature larval hosts (Table I). When the latter emerged as adults, 14 (19 per cent) of 74 individuals showed the typical integumentary defects. Here again, the local inhibition of metamorphosis was conditional upon the close proximity of implant and overlying cuticle. Control animals received the anterior third from which the brain-ring gland complex had been removed. Only 5 per cent of these animals showed the typical defect. This could be due to the retention of the ring gland by some of the anteriors since the ring gland grasps the pharynx rather tenaciously. As a final control imaginal discs from mature larvae were implanted into hosts of the same age, and none of these hosts showed any abnormalities.

These studies, coupled with Vogt's (1946) results, show that the ring gland of adult *Drosophila* and the brain-ring gland complex of first instar *Drosophila*

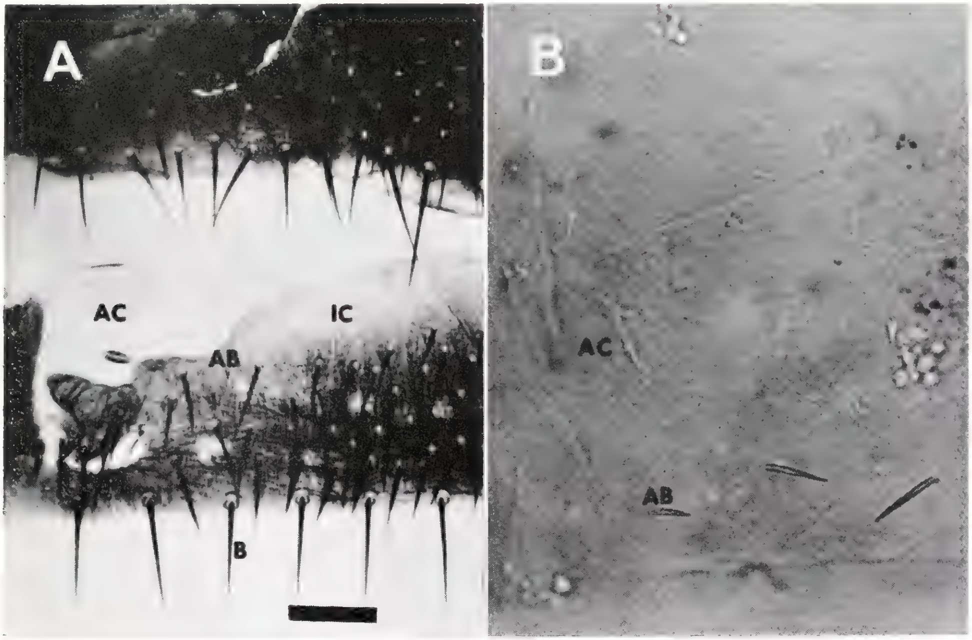

FIGURE 2. Effect of Cecropia juvenile hormone on the adult differentiation of the abdomen of *Drosophila;* A., 0.05 μg/animal; B., 5 μg/animal. Symbols used are: AB., abnormal bristles; AC., aberrant cuticle; B., normal bristle; IC., imaginal cuticle. Compare AC to Figure 1C. Scale bar equals 100 μ.

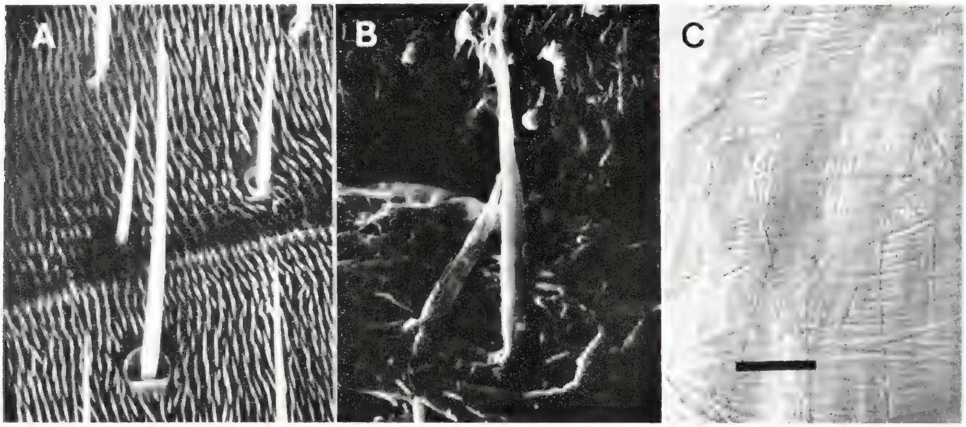

FIGURE 3. Scanning electron micrographs of: A., acetone treated control adult cuticle; B., juvenile hormone treated cuticle; C., pupal cuticle. Scale bar equals 20 μ.

cause local inhibition of abdominal metamorphosis. The aberrant cuticle is similar to pupal cuticle in that both lack pigment, bristles and hairs. In addition, surrounding the pupal-like regions are areas which have short, nicked, stubby or incompletely pigmented bristles.

The molting of the implants in these studies, published elsewhere (Postlethwait, 1973), confirm Bodenstein's (1944) results and show that larval tissues must develop competence to respond appropriately to the environment at metamorphosis.

Effect of exogenously supplied JH on the metamorphosis of Drosophila

Having ascertained the morphogenetic defects produced by implants containing juvenile endocrine organs, we centered further attention on the effects of topically applied, synthetic JH. Individuals receiving in excess of 0.05 μg C18JH formed defective pharate adults which failed to emerge. As illustrated in Figures 2 and 3, the abdominal tergites of individuals receiving low doses of JH showed patches of cuticle identical to those produced by the implantation of brain-ring gland complexes. In the case of individuals treated with the highest doses, the entire "adult" abdomen was covered by an aberrant cuticle showing an almost complete suppression of pigmentation and of bristles and hairs (Fig. 2B). The cuticle in the affected areas was impressively different from that of the controls (Fig. 1) and in many cases was indistinguishable from pupal cuticle over large areas. Although the aberrant cuticle could not be distinguished from pupal cuticle in whole mounts, it must be noted that the normal pupal cuticle lacks any projections or irregularities which in unstained whole mounts permit its positive and unambiguous identification.

But we can conclude that exogenously supplied synthetic JH causes a general syndrome identical to that produced locally by an implanted active young larval brain-ring gland complex, and in both cases the aberrant cuticle is indistinguishable from pupal cuticle.

Developmental stages during metamorphosis which are most sensitive to exogenously supplied JH

Effects on adult eclosion. Figure 4A records the percentage of flies which failed to eclose when specific doses of C18JH were administered at discrete ages ranging from the mid-third instar until one day after the initiation of pupariation. All except the lowest dose had major effects in blocking eclosion when adminis-

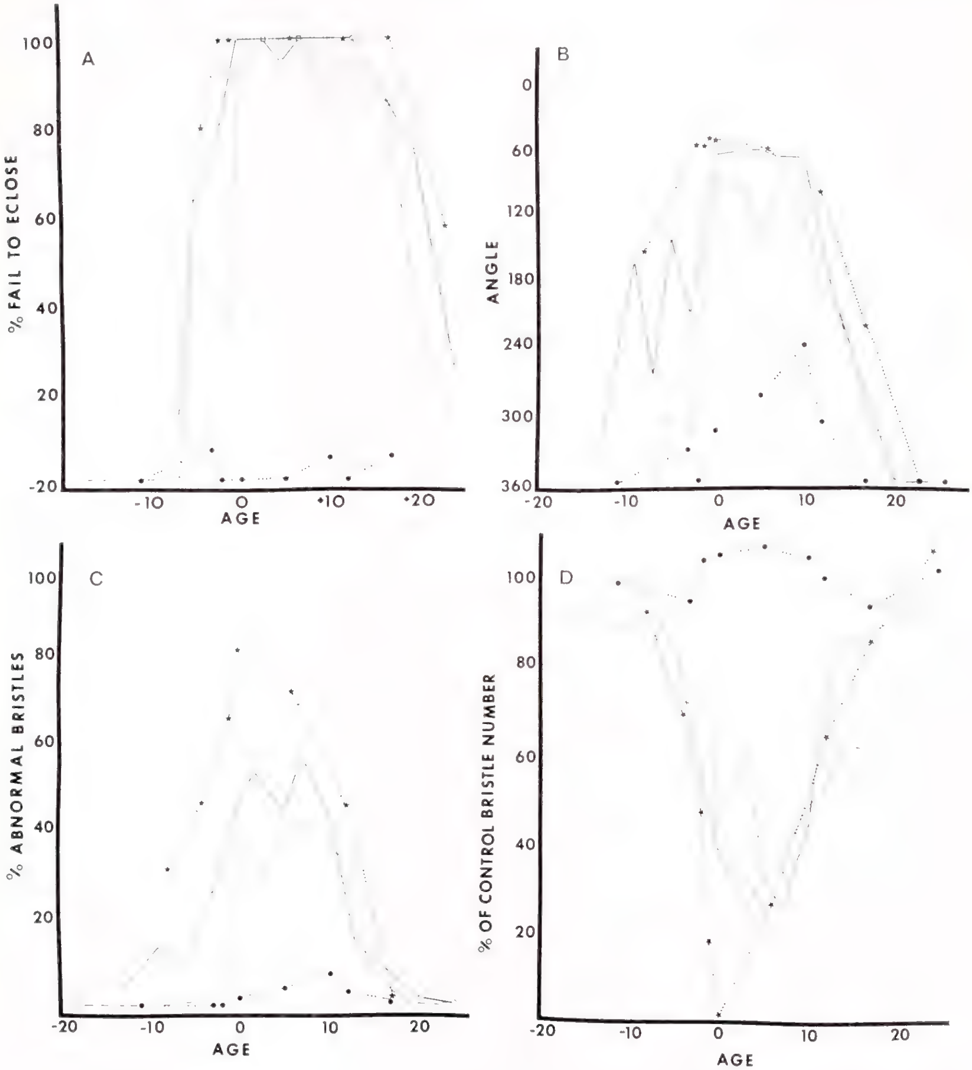

FIGURE 4. Four parameters of JH activity and their relationship to age at time of treatment; A., per cent failing to eclose *vs.* age; B., failure of male genitalia to rotate; C., per cent abnormal bristles; D., per cent of bristles missing on the male 5th tergite and female 6th tergite; Stars, 5.0 μg; open circles, 0.5 μg; squares, 0.05 μg; filled circles, 0.005 μg.

tered during a certain critical period. The latter begins about 5 hours prior to the onset of pupariation and persists for approximately 25 hours thereafter. Maximal sensitivity includes the first 12 hours after pupariation.

Effects on the rotation of the male genitalia. During a terminal phase of adult differentiation of Dipterous insects the male genitalia undergo a permanent rotation with respect to the long axis of the body (Scudder, 1971). In the lower Diptera the rotation is through 180°; in the higher Diptera such as *Drosophila*, it is through 360° (Gleichauf, 1936). Genitalia rotation is partially or completely blocked in mosquitoes (Spielman and Williams, 1966) and *Sarcophaga* (Bhaskaran, 1972) derived from pupae exposed to JH analogues; the same is true for *Drosophila* according to Bryant and Sang (1968) and Madhavan (1973).

These findings are confirmed in the present study. Thus, in Figure 4B, is recorded as a function of dosage and time of application the per cent by which the male genitalia failed to undergo the normal 360° rotation. Here again, the period of maximal sensitivity is the first 12 hours after the initiation of pupariation.

Effects on bristle number and morphology. In order to quantify the inhibition of metamorphosis, a detailed study on the 5th abdominal tergite of males and the 6th of females was undertaken. These segments were chosen because they have both bristles and hairs (trichomes) and because the posterior tergites are affected to a greater degree than the anterior tergites. The male 5th tergite is characterized by 54 ± 4 bristles; the female 6th tergite by 62 ± 3 bristles in normal flies or in controls treated only with acetone. Counts were made of the total number of bristles which had differentiated on these tergites, as well as the number which were abnormal.

Figure 4D records the percentage of bristles (with reference to control values) as a function of dose and time of application. In Figure 4C is plotted the percentage of abnormal bristles among those which had differentiated. Here again, we see that the effects are dose-dependent and that the sensitive period is from shortly before to about 15 hours after the initiation of pupariation.

Effect of dose and site of application on integumentary defects

Topical application of the JH doses employed was not able to suppress the normal adult differentiation of the integument of the head or thorax. Only an occasional individual which had received the highest dose (5 μg) at the most sensitive stage showed a few abnormal bristles on the head, but no other trace of abnormal characters on either the head or thorax. By contrast, the adult differentiation of the abdomen was subject to inhibition by JH when the latter was applied during the sensitive period. The typical result was the formation of more or less extensive zones of unpigmented, hairless cuticle in which the bristles were either missing or of aberrant size and shape. Featureless zones within this cuticle were indistinguishable from pupal cuticle as shown above. These characteristics are illustrated in Figures 5B–5E in the case of four individuals that received graded doses of JH in the "white puparium" stage. For comparison, a normal adult integument is depicted in Figure 5A and a normal pupal integument in 5F.

In the experiments summarized in Figure 6, graded doses of C18-JH were applied to individuals at a single highly sensitive stage—namely, at the outset of

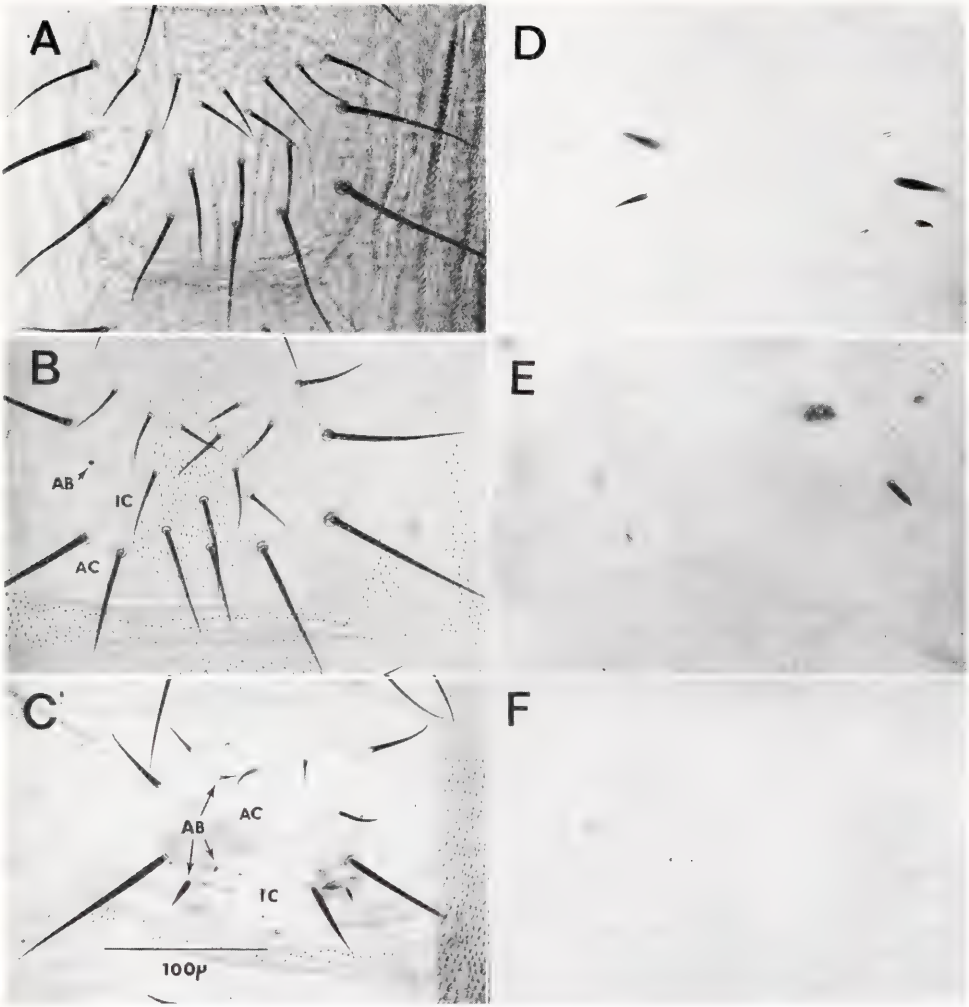

FIGURE 5. Affect of increasing doses of C18JH on the sternites of *Drosophila;* A., acetone-treated control; B., 0.002 µg; C., 0.005 µg; D., 0.05 µg; E., 0.5 µg; F., Pupal cuticle; AB, abnormal bristles; AC, aberrant cuticle; IC, imaginal cuticle.

pupariation (white puparia). The several parameters of juvenilization are in each case plotted as a function of the logarithm of dose. Table II records the threshold doses, the saturating doses, and the doses that give 50% effects.

When low doses of hormone were administered during the critical period, the posterior abdominal segments were more sensitive than the anterior segments and the abdominal sternites were routinely more effected than the tergites. Meanwhile, as mentioned above, the head and thorax remained insensitive at all stages.

The insensitivity of the head and thorax was examined in two series of experiments. In the first of these the hormone was applied to the anterior ends of white prepupae rather than to the posterior ends. This shift in the site of application

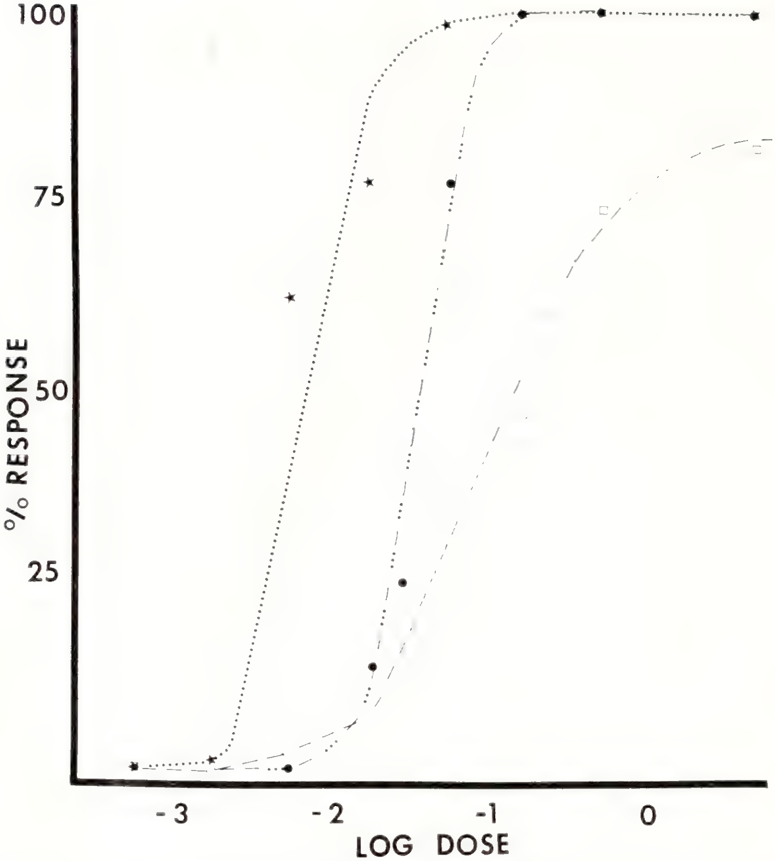

FIGURE 6. Dose response curves; stars, abnormal sternite bristles; filled circles, failure to eclose; squares, abnormal tergite bristles.

had no effect on the differentiation of the adult head and thorax. Moreover, the effect on the abdomens was indistinguishable in the two cases except that the rotation of the male genitalia was somewhat more inhibited in the individuals that received the hormone at the hind end.

TABLE II

Quantitative aspects of the action of C18 JH on white puparia

Effect	(Critical doses (μg/individual)*		
	Threshold	50% Effect	100% Effect
Abnormal bristles on sternites	0.002	0.006	0.05
Failure to eclose	0.016	0.03	0.2
Inhibition of rotation of genitalia	0.005	0.03	0.2
Abnormal bristles on tergites	0.005	0.6	0.75

* Each dose was topically applied in 0.3 μl acetone.

TABLE III

Relative abilities of JH analogues to block metamorphosis

Approximate rank	Analogue Number	Designation	Concentration μg/g	# of animals tested	% fail to eclose	% with morphogenetic effect on sternites	Degrees male genitalia rotated	% control bristle #	% aberrant bristles per tergite
1	XIII	epoxy geranyl sesamole	34	28	100	100	15 ± 12	36 ± 18	95 ± 10
2	VIII	iso-C17-JH	34	15	87	100	54 ± 40	70 ± 17	55 ± 20
3	XXII	ZR-515	34	22	100	100	100 ± 33	66 ± 21	65 ± 34
4	IX	C16-JH	34	15	60	100	67 ± 36	90 ± 12	39 ± 20
5	I	C18-JH	34	47	76	100	87 ± 39	82 ± 15	36 ± 16
	I	C18-JH	17	15	27	100	240 ± 100	97 ± 13	19 ± 13
6	I + XXI	C18-JH + sesamex	17 17	15	33	100	200 ± 70	103 ± 10	13 ± 7
7	VI	C17-JH	34	15	47	100	154 ± 52	91 ± 15	18 ± 15
8	XV	R-20458	34	15	60	100	154 ± 79	102 ± 10	19 ± 9
9	XVI	R-19828	34	15	93	100	300 ± 42	104 ± 14	8 ± 3
10	XIV	geranyl sesamole	34	15	67	100	283 ± 67	104 ± 10	5 ± 4
11	XII	ClMe-C16-JH	34	13	0	100	265 ± 86	108 ± 11	4 ± 4
12	XI	C16-JH-S	34	15	0	100	308 ± 66	110 ± 11	4 ± 5
13	III	MBHF	34	15	0	100	342 ± 40	103 ± 14	1 ± 2
14	XX	Williams-Law	34	15	0	78	360 ± 0	107 ± 11	0.4 ± 1
15	XIX	EDCF	34	15	0	29	360 ± 0	102 ± 11	0.2 ± 0.8
16	IV	Farnesenate	34	14	0	43	356 ± 11	102 ± 10	0.1 ± 0.5
17	V	Farnesol	34	14	0	50	360 ± 0	102 ± 9	0 ± 0
18	II	C18-JH-N	34	14	7	14	360 ± 0	108 ± 10	0.1 ± 0.1
19	X	C16-JH-N	34	14	0	0	360 ± 0	110 ± 8	0 ± 0
20	VII	C17-JH-aldehyde	34	15	0	0	360 ± 0	98 ± 7	0 ± 0
21	XVII	Epoxy geranyl methyl benzoate	34	14	0	0	360 ± 0	102 ± 9	0 ± 0
22	XVIII	geranyl methyl benzoate	34	10	0	0	360 ± 0	98 ± 12	0 ± 0
23	XXI	Sesamex	34	15	0	0	360 ± 0	100 ± 12	0 ± 0
24		Acetone		26	0	0	360 ± 0	100 ± 9	0 ± 0

In the second series of experiments the operculum was removed from puparia and the hormone was applied directly to the pupal head. (This operation cannot be carried out before the 12th hour since, prior to that time, the epidermis is firmly attached to the operculum.) In each of the treated individuals the head and thorax underwent essentially normal adult development. A few bristles were bent abnormally, but this occurred also on animals whose operculum was removed and who were then treated with acetone. The effects on the abdomen could not be distinguished from that seen in controls in which the hormone was applied to intact puparia of the same age.

The relative abilities of different JH analogues to block metamorphosis

As a check on the general types of molecules which have JH activity in *Drosophila*, I compared the morphogenetic effects of 22 different JH analogues (Table III). The conclusions drawn below are subject to the fact that the isomer composition of most of the compounds tested is unknown. The study shows that for *Drosophila* the synthetic JH analogues epoxy geranyl sesamole and ZR-515 are both more active than any of the hormones isolated from Lepidoptera (compounds I, VI and IX, Roller, Dahm, Sweely and Trost, 1967; Meyer, Schneiderman, Hanzmann and Ko, 1968; Dahm and Roller, 1970; Judy, Schooley, Dunhan, Hall, Bergot and Siddall, 1973). A comparison of JH analogue activities shows that for *Drosophila* a methyl group at position 11 is better than ethyl since iso-C17-JH (VIII) is more active than C18-JH (I), and C16-JH(IX) is more active than C17-JH (VI). An epoxy ring is better than a double bond between carbons 10 and 11 since C18-JH (I) is more active than methyl bishomofarnesoate (III) and epoxy geranyl sesamole (XIII) is more active than geranyl sesamole (XIV). An epoxy ring is better than an imino ring or a thio ring between carbons 10 and 11 since C16-JH (IX) is more active than C16-JH-aziridine (X) or C-16-JH-episulfide (XI) and C18-JH (I) is more active than C18-JH-aziridine (II). An ethyl group at position 7 is better than a methyl since iso-C17-JH (VIII) is more active than C16-JH (IX) and C18-JH (I) is more active than C17-JH (VI). A methyl group at position 7 is better than a chloromethyl since C16-JH (IX) is more active than chloromethyl-C16-JH (XII). Sesamole is more active than other modified benzenes in an ether link to geranol since epoxy geranyl sesamole (XIII) is more active than R-20458 (XV), R-19828 (XVI) or epoxy geranyl methyl benzoate (XVII).

Discussion

These experiments show that a characteristic syndrome of abdominal cuticular abnormalities can be induced in *Drosophila* by either implanting a first instar brain-ring gland complex into a mature larva, or by topically treating a freshly pupariated *Drosophila* with Cecropia juvenile hormone. The characteristics of the aberrant cuticle involve a failure of the cuticle to pigment, or to form cell hairs. Surrounding these areas are bristles which are short, nicked, or incompletely metamorphosed. The bristle irregularities confirm the results of earlier workers (Dearden, 1964; Bryant and Sang, 1968, 1969; Ashburner, 1970b; Madhavan, 1973).

Since the entire brain-ring gland complex (including several endocrine organs) was transplanted, the precise inducer of the aberrant cuticle is in question. But the evidence is consistent with the interpretation that the corpus allatum part of the ring gland inhibits metamorphosis. First, either ring glands or brain-ring gland complexes of *Calliphora* when transplanted into fifth instar hosts of *Rhodnius* induce the retention of larval cuticle in the adult (Wigglesworth, 1954). Secondly, adult corpora allata implanted into mature larval hosts inhibit normal adult development of the abdomen in *Drosophila hydei* (Vogt, 1946) or *Calliphora* (Possompes, 1953), causing the same syndrome as appeared in our experiments. Thirdly, first instar ring glands of *D. hydei* implanted into adult females which had been deprived of their ring glands induced vitellogenesis, whereas brains alone failed to induce egg maturation (Vogt, 1943). It therefore seems that the ring gland of flies produces a juvenile hormone, and it may be that transplanted young ring glands caused the aberrant cuticle in our transplants.

The experimentally induced aberrant cuticle was indistinguishable from pupal cuticle, but that does not prove that it *is* pupal cuticle. Since pupal cuticle is devoid of unique and specific cuticular markers, its positive identification using purely morphological criteria is frustrated.

Five higher flies other than *Drosophila* have been tested for their response to juvenile hormone analogues: *Sarcophaga bullata* (Srivastava and Gilbert, 1968, 1969; Bhaskaran, 1972), *Sarcophaga argyrostoma* (Fraenkel and Hisao, 1970), *Calliphora erythrocephala* (Pihan and Bautz, 1970), *Stomoxys calcitrans* (Wright, 1970; Wright and Rushing, 1973; Wright, Crookshank and Rushing, 1973), and *Musca domestica* (Herzog and Monroe, 1972; Cerf and Georghiou, 1972). These studies showed that JH analogues inhibited eclosion and metamorphosis and suppressed the differentiation of hairs, bristles and pigment characteristic of the adult fly. In three of these investigations histological sections were prepared. Srivastava and Gilbert (1968, 1969) interpreted their sections as showing two pupal cuticles, the second apparently being induced by JH. Wright (1970) and Bhaskaran (1972) found no second pupal cuticle nor a pupal-adult intermediate cuticle. Bhaskaran (1972) suggested that the "second pupal cuticle" described by Srivastava and Gilbert (1968, 1969) may be undifferentiated adult cuticle or the undigested inner layers of the first pupal cuticle. The latter explanation could not hold for the pupal-similar cuticle reported here for *Drosophila* since the normal and aberrant cuticle are contiguous in treated abdomens. The aberrant cuticle in *Drosophila* may be merely undifferentiated adult cuticle which, by chance, is indistinguishable from pupal cuticle. The available data do not allow us to distinguish between the possibilities that in *Drosophila* juvenile endocrine glands and synthetic Cecropia JH induce a second pupal cuticle in the abdomen, or rather an aberrant adult cuticle that cannot be distinguished from pupal cuticle in whole mounts. Biochemical or immunological identification of pupal-specific cuticular protein could resolve the dilemma.

The procedure developed here provides a simple quantitative method for the assay on *Drosophila* of substances suspected to possess JH activity. This test is a sensitive measure of JH activity—as little a 0.0015 μg C18-JH can be detected. The dose-response curves show that for the posterior three sternites the dose resulting in 50% morphological inhibition of metamorphosis (ID_{50}Morph., Slama, 1971) is 5 μg/g live weight. The ID_{50}Morph. for topically applied JH is about 8 μg/g for Tenebrio, and 25 μg/g for *Pyrrhocoris* (Slama, 1971; Williams, 1970). It therefore appears that the *Drosophlia* abdomen is about as sensitive to JH as other insects.

In many insect orders the male genitalia rotate to accommodate the female during copulation. In Diptera the rotation occurs during development and is permanent. This rotation involves the abdominal tissue surrounding the genitalia rather than the genitalia *per se* (Gleichauf., 1936; Scudder, 1971). One of the effects noted after JH application to *Drosophila* is failure of the male genitalia to complete rotation (Bryant and Sang, 1968). Failure to complete rotation occurs also after treatment of the mosquito *Aedes aegypti* (Spielman and Williams, 1966) and *Sarcophaga* (Bhaskavan, 1972) with JH analogues. The JH sensitive stage for the rotation in *Drosophila* is from shortly before pupariation until about 15 hours (Fig. 5B). This sensitive phase is well prior to the actual time of rotation for *Drosophila*, which does not occur until the late stages of metamorphosis (Gleichauf, 1936).

These results confirm that the abdomen is quite sensitive to JH while the head and thorax are not and the differentiation of the genitalia is less sensitive than the abdomen (Dearden, 1964; Bryant and Sang, 1969; Ashburner, 1970b; Madhavan, 1973). This difference is probably not due to the method of application since treatment on the anterior or posterior gives similar results and removal of the operculum to allow application of hormone directly to the pupal head does not cause the head and thorax to be affected. In addition, feeding the hormone after the method of Bryant and Sang (1969) elicits the same syndrome of abnormalilities which are described here for topical application.

If the difference in response of different tissues is not an artefact it must have some basis in the developmental physiology of various body parts. The development of the epidermis of the imaginal abdomen differs from the development of the head, thorax, and genitalia in two significant respects. First, the adult abdomen of higher flies arises from clusters of histoblasts, which undergo little or no cell division from embryogenesis until the time of pupariation (Robertson, 1936; Garcia-Bellido and Merriam, 1971a; Guerra, Postlethwait and Schneiderman, 1973). The other integumentary structures arise during embryogenesis as sacs of cells, called imaginal discs. The imaginal disc cells proliferate rapidly during larval life, but slow down after pupariation (Becker, 1957; Bryant and Schneiderman, 1969; Bryant, 1970; Garcia-Bellido and Merriam, 1971b; Postlethwait and Schneiderman, 1971; Ulrich, 1971). Thus, when cell division rate is high in the abdomen, cell division rate is low in the other imaginal anlagen. Our results (Fig. 5) show that the time of JH sensitivity for the abdomen is precisely the time of rapid proliferation of abdominal histoblast cells.

A correspondence of JH sensitivity and high proliferation rate appears to be a general phenomenon in the insect integument. For example, in *Rhodnius*, sensitivity to exogenously supplied JH analogues is greatest at 8 days after feeding, which is the time mitosis is beginning in the epidermis (Wigglesworth, 1963). In some cases an increased proliferation rate can be induced experimentally. Piepho and Heims (1952) demonstrated that regenerating cells of the waxmoth *Galleria mellonella* are much more sensitive to JH than undividing cells. This fact has been exploited in the very sensitive "wax test" for JH activity (Schneiderman and Gilbert, 1958).

The second main difference between abdominal and anterior tissues is that the pupal cuticle of the abdomen is secreted at least largely by the larval abdominal cells, while the pupal cuticle of the anterior is secreted by the imaginal disc cells. So it is not clear whether the aberrant (pupal-like) adult cuticle in *Drosophila* is secreted by larval or imaginal cells. It is clear for *Sarcophaga* that the aberrant adult cuticle is secreted by imaginal cells (Bhaskaran, 1971). Further histological studies are in progress to answer this question for *Drosophila*.

The correspondence between cell division and JH activity has been thought to be fundamental to the action of the hormone. Williams and Kafatos (1971) have proposed that cell division in the presence of JH delays the activation of new gene sets, whereas cell division in the absence of JH permits new programs of genetic information to be utilized. Perhaps further hormonal research on *Drosophila*, in which genetics can be effectively used to investigate developmental problems, will lead to a better understanding of such problems.

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SUMMARY

Topical application of Cecropia juvenile hormone (JH) blocked metamorphosis of the abdominal integument of pupating *Drosophila* larvae. JH treated adult flies retained more or less extensive areas of cuticle which could not be distinguished from pupal cuticle in whole mounts. Implantation into mature larvae of fragments of first instar larvae containing brains and ring glands provoked localized retention of patches of incompletely metamorphosed cuticle. An assay procedure for compounds with juvenile hormone activity was developed by defining the critical developmental stage and dose-response relationships. Twenty-two substances suspected of having juvenile hormone activity were assayed for their effect on *Drosophila*. These findings support the choice of *Drosophila* for resolving the genetic basis of juvenile hormone action.

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HARVEST OF PLANKTONIC MARINE ALGAE BY CENTRIFUGATION INTO GRADIENTS OF SILICA IN THE CF-6 CONTINUOUS-FLOW ZONAL ROTOR[1]

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The harvest of the more common algae from laboratory cultures rarely presents serious problems. However, the collection (or concentration) of living algal cells in good physiological condition from dilute cultures or from natural plankton populations is much more difficult. The two major devices commonly used are filtration and centrifugation. Filtration is carried out commonly on membranes of modified cellulose (Clarke and Sigler, 1963), with the aid of a suction pump. The greatest advantage of this method as a concentrating device is that it is able to collect microalgae or cells of very low density. However, concentration by filtration is limited to small volumes and leads to the eventual clogging of the filter by the packed cells when vacuum is applied.

Several methods have been devised which avoid these problems. One involves the use of a reverse-flow vacuum (Dodson and Thomas, 1964) in which the pressure operates from above, making the process more gentle and avoiding the packing of cells. This method itself has been modified to allow a relatively large volume of water to be concentrated in a short period of time (20 liters to 300 ml in 3 hours) (Holm-Hansen, Packard and Pomeroy, 1970). A second process uses a direct vacuum but involves a stirring blade in the flask above the filter which prevents the particles from settling at all during the concentration process (Morris and Yentch, 1972).

Continuous-flow centrifugation with the classical Foerst rotor or the Szent-Gyorgyi-Blum modification of the Sorvall rotor is another widely used method. This method is reasonably efficient, but sensitive algal cells may be damaged by pelleting against the rotor wall and the method is essentially unselective; all particles with a sedimentation rate above some limiting value will be collected.

The variant on zonal centrifugation known as continuous sample-flow with isopycnic banding (*cf.* Cline and Ryel, 1971) offers a number of theoretical advantages in the concentration (and simultaneous purification) of particles: large capacity, more efficient recovery at substantially lower speeds than are required for conventional continuous-flow centrifugation, and avoidance of pelleting. Plankton, including algae, have been collected in sucrose gradients in the B-XVI and K-II zonal rotors (Lammers, 1971), but we do not know either the efficiency of recovery or the integrity of the recovered algae.

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TABLE I

Algae investigated

Species	Clone	Class	Approximate dimensions	Origin
Dunaliella tertiolecta	Dun	Chlorophyceae	6.5 × 10 μ	Unknown; possibly derived from Plymouth #83
Pyramimonas sp.	Pyr-I	Prasinophyceae	8 × 15 μ	San Francisco, California
Thalassiosira fluviatilis	Actin	Bacillariophyceae	20 μ (isodiametric)	Long Island Sound (Gardiner's Island)
Synechococcus bacillaris	Syn	Cyanophyceae	0.75 × 1.5 μ	Milford, Connecticut

In any event it should be possible to harvest algae with centrifuge systems which are much slower and less costly than the zonal centrifuges designed for the purification of viruses. We have, therefore, tested an alternative means of harvesting algal plankton in the CF-6 rotor (Casciato, 1973), a continuous-flow zonal rotor.

MATERIALS AND METHODS

Organisms and conditions of growth

The algal strains used in this work are listed in Table I, together with clonal designation, algal class, origin, and approximate size of each. Algae will be referred to by clonal designation in this paper. All clones are maintained by R. R. L. Guillard at the Woods Hole Oceanographic Institution. These particular species are taxonomically varied and cover a range of sizes, though all are in the smallest size class of the phytoplankton (the nannoplankton). Two clones (Dun and Pyr-I) are naked flagellates; the blue-green algal clone Syn has a multilayered wall with protein, carbohydrate, and mucopeptide components; the diatom Actin has a wall composed of a silica shell in addition to an organic matrix. We expected the structural variability to influence the density during centrifugation. The chlorophyte Dun was particularly useful because of its motility and general hardiness; a visual indication of its viability under gradient conditions and manipulation could be obtained by observing motility.

All algae were grown in an enriched articificial seawater. A seawater base was made by using the major constituents ($MgSO_4$, $MgCl_2$, $CaCl_2$, KCl, $NaHCO_3$, and NaCl) of ASP-M (McLachlan, 1964) plus 1 ml/l of an 0.6% (w/v) solution of H_3BO_3. This seawater base was then supplied with the nutrients of enrichment "f" (Guillard and Ryther, 1962) with all nutrients at half the concentrations specified.

Cultures were grown at 20–24°C under continuous illumination of 3500–6000 lux from cool-white (Sylvania Co.) fluorescent lamps. Cultures were grown in Erlenmeyer flasks or carboys with aeration.

Preparation of gradients

The silica gradients were prepared as follows: Ludox AM (E. I. du Pont de Nemours and Co., Wilmington, Delaware), a preparation of colloidal silica with partial substitution of Si by Al, was allowed to dialyze against distilled water for at least two weeks; water was changed daily. The Ludox was then collected and used for several runs. The reason for this dialysis is that, contrary to the evidence of others with animal cells (Mateyko and Kopac, 1963; Pertoft, 1969), Ludox AM was found to be highly toxic to algal cells, marine algae as well as *Euglena gracilis*. We suspect that the toxicity is due to the presence of a biocide in the commercial product. Two weeks of dialysis does not completely remove the toxic quality

The gradients used were: 40% to 70% v/v dialyzed Ludox for Dun, Pyr-I, Syn and 20% to 50% v/v dialyzed Ludox for Actin (see graphs in Results). Gradients of zero to 3% w/v NaCl ran counter to the Ludox to minimize gelling. The appropriate gradients were established by trial runs with step gradients in swinging buckets. The banding densities were monitored by the use of calibrated density beads (Clark Wilcox and Assoc., Los Altos, California). Gradients were also prepared from mixtures of Ficoll in seawater. Ficoll is a trade name for polysucrose, available from Pharmacia Fine Chemicals, New Market, New Jersey.

Harvest and centrifugation

Cells were harvested in the 620-ml CF-6 zonal rotor (IEC/Damon, Needham Heights, Massachusetts), in the PR-6 (IEC) centrifuge. The gradient consisted of 235 ml of starting solution, 220 ml of limiting solution and 115 ml of underlay. The starting and limiting solutions were mixed in the IEC gradient former and pump (#3651) to give a gradient which was linear with volume, and were pumped into the rotor at 40 ml/min. The rotor speed during loading was 1500 rpm. The sample was loaded at 100 or 200 ml/min by either the Harvard (#600–00) or the Cole-Parmer Masterflex (#WZ-1R031) peristaltic pump. The sample was then run for 15 min at 4000 rpm to band the particles, slowed to 1000 rpm, and the gradient displaced by pumping at 35 ml/min. The gradient was collected in 30-ml fractions in chilled graduated test tubes.

In order to study the efficiency of collection, samples of the algal suspensions were fixed with 1% glutaraldehyde and the cell concentrations determined subsequently in a Coulter Counter by Dr. J.-J. Morgenthaler. Background counts were taken for comparison with counts from supernatants and original culture samples (see Table II).

Analyses

Growth. Relative cell concentration was measured by fluorometry of the chlorophyll (Knight, 1968). A 436 nm interference primary filter and a #66 Klett secondary filter were used in a Turner Model III fluorometer. Fluorescence was found to be proportional to cell number. Daily samples of about 4 ml were taken aseptically from the cultures in 250-ml culture flasks. Where dilution was necessary, it was done with artificial seawater. The blank was also artificial seawater. Growth rates and progress curves were obtained by plotting relative fluorescence against time on a semilog scale (Fig. 3 a–d).

Chlorophyll. The concentrations of cell material within the peak zones of the gradient were established by measuring the amounts of chlorophyll spectrophotometrically. This method was also used to compare the chlorophyll content of the original culture suspension with that of the harvested cells. One milliliter from a 30-ml gradient fraction or 100 ml of the culture suspension concentrated to 1 ml by centrifugation were placed in 4 ml of 100% acetone and allowed to remain in the cold and dark for 1/2 hour. They were then centrifuged at 2000 rpm for 10 min and the absorbance of the supernatant read at 652 nm (Arnon, 1949; Bruinsma, 1961).

Polarography. Oxygen polarography was used to determine the rates of photosynthesis and respiration of harvested and unharvested cells. Harvested cells from the tube corresponding to the peak concentration of cells were diluted 1 : 10 or 1 : 5 in artificial seawater or 100 ml of unharvested cells were centrifuged at 2000 rpm for 10 min and resuspended in 3 ml of medium. Oxygen exchange by the algal suspensions in the dark (respiration) and in the light (net photosynthesis) were measured with the Model 53 Biological Oxygen Monitor (Yellow Springs Instrument Company, Yellow Springs, Ohio).

RESULTS

Efficiency of collection

The efficiency of collection of algae by the rotor was measured by comparing the concentration of algae in the original suspension with that in the effluent from the rotor. The four organisms studied cover a range of sizes that include most planktonic forms. The clean-out at any given speed is a function of the size of the alga (Table II). On this basis we have projected the flow rates required to yield ≥ 90% clean-out. For the larger algae at 1500 rpm and 200 ml/min, clean-out was essentially quantitative, and flow rates can probably be increased severalfold without significant decrease in clean-out. For *Synechococcus*, which is of bacterial size, the clean-out was no better than 78% at maximum rotor speed and at the lower flow rate. *Synechococcus* may be at the lower limit of particle

TABLE II

Clean-out of algal suspensions in the CF-6. Fractional "clean-out" is a measure of the efficiency of retention of particles in a continuous-flow rotor and defined as

$$\left[\frac{\text{original concentration} - \text{effluent concentration}}{\text{original concentration}}\right]$$

The coefficient of variation of clean-out varied from 0.2 to 8%

Organism		Flow-rate ml/min	Cell number/ml		Fractional clean-out
			Initial	Effluent	
Dun	6.5 × 10 μ	200	137,767	968	0.99
Actin	10–20 μ	200	50,160	111	0.99
Pyr-I	8 × 15 μ	200	71,585	269	0.99
Syn*	0.75 × 1.5 μ	100	8,430	1818	0.78
		150	8,430	1790	0.78
		200	8,430	3115	0.63

* Determined at rotor speed of 6000 rpm. All others at 1500 rpm.

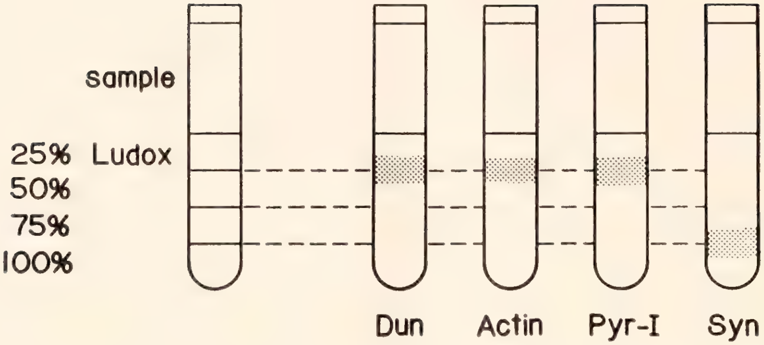

FIGURE 1. Diagram of isopycnic banding of different algae in step gradients of Ludox.

size that can be separated under the present conditions. Very few algal species are as small as *S. bacillaris*.

Equilibrium densities

The banding densities were initially estimated in step gradients of silica in swinging buckets. After centrifugation at 5000 rpm for 5 min, the banding patterns sketched in Figure 1 were obtained. These measurements showed that Dun, Actin and Pyr-I banded at approximately the same densities, while Syn banded at a much higher density.

More precise distributions were determined from direct measurements in the continuous gradients recovered from the CF-6. The volumes in which the cells were recovered were in the order of 100 ml (Fig. 2 a–d), but substantial heterogeneity in density was sometimes observed. Dun banded between $\rho = 1.07–1.12$, but principally near 1.08. Actin banded between 1.09–1.12.

Integrity

Any method of harvest is of limited usefulness if the cells cannot be recovered physiologically intact and viable.

We tested physiological state by polarographic measurements of respiration and photosynthesis, before and after harvest, for some of the algae. A summary of the data is presented in Table III. In the case of Dun, there was no loss of photosynthetic or respiratory activity after banding in Ludox gradients. For the other species, both the rates of photosynthesis and respiration were decreased to 1/3 to 1/5 of the original values after the cells were harvested from the Ludox gradients. To determine whether Ludox itself affected photosynthesis or respiration, separate experiments were run whereby polarography was measured on cells in the absence or presence of the silica sol ($\simeq 66\%$ Ludox) using the organisms Dun and Syn. In both cases there was an enhancement (doubling) of respiration in the presence of Ludox. However, photosynthesis was again lowered to about 1/4 in the case of Syn and essentially unchanged in the case of Dun, in the presence of Ludox. There have been reports that Ludox catalyzes certain biological

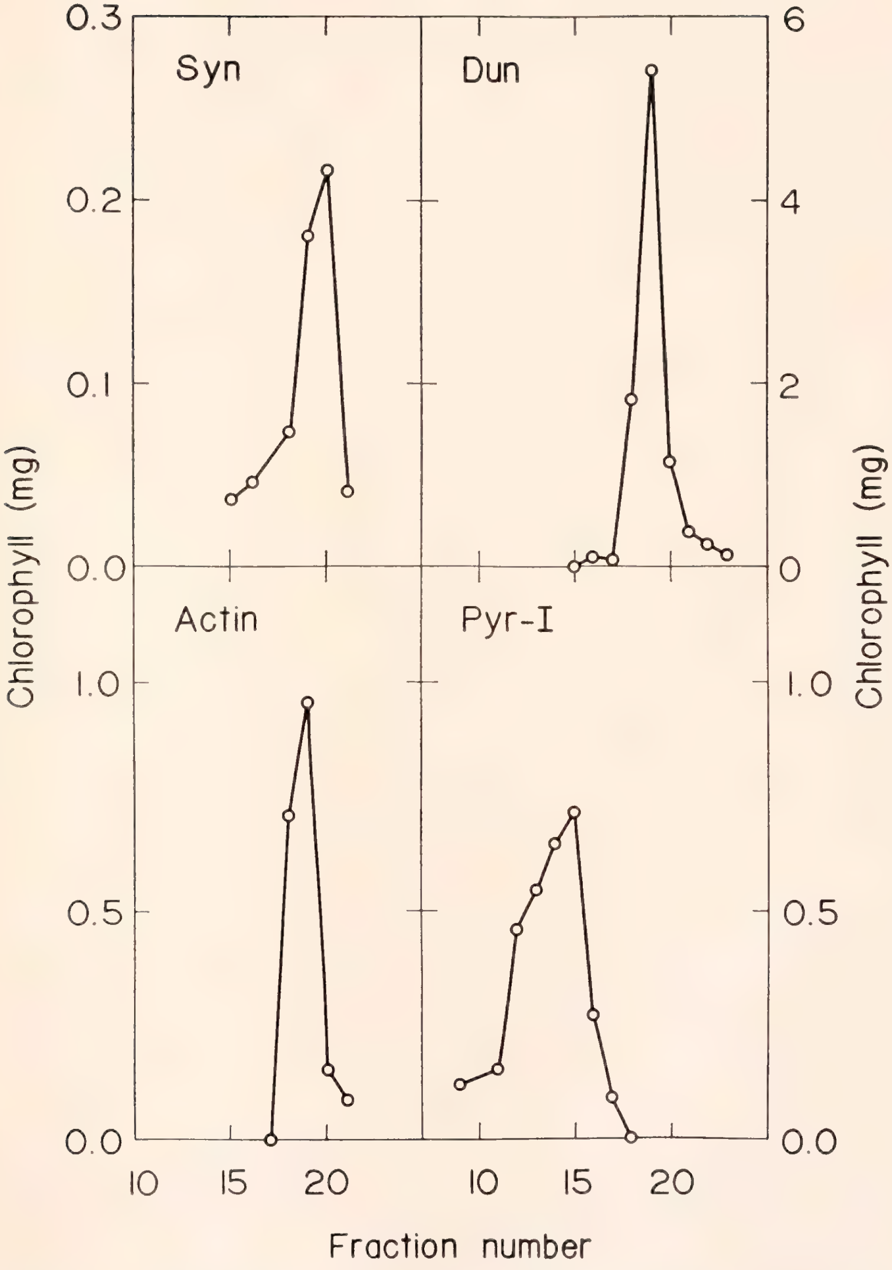

FIGURE 2. Recovery of algae after continuous sample flow with isopycnic banding in gradients of Ludox. Details of the method are described in the text. Cell concentration was estimated by monitoring the absorbance of the fractions at 652 nm; *Synechococcus bacillaris* (Syn); *Dunaliella tertiolecta* (Dun.); *Thalassiosira fluviatilis* (Actin); *Pyramimonas* sp. (Pyr-I).

TABLE III

Respiration and photosynthesis of algae before and after centrifugation through Ludox gradients

Sample	Rates of O_2 exchange nmoles O_2 $hr^{-1} \cdot \mu g$ chlorophyll^{-1}	
	Dark	Light
Pyr-I (83/52)		
Original	− 61.5	+ 21
Ludox-harvested	− 23.2	+ 7.8
Syn (83/54)		
Original	−229	+285
Ludox-harvested	− 89	+ 52
Dun (83/57–SB)		
Original	− 12.6	+136
Ludox-harvested	− 12.6	+210

oxidations (Slawson, Adamson and Mead, 1973). This may account for the observed increase in O_2 uptake by the cells in the presence of concentrated amounts of Ludox.

Viability was measured by inoculating fresh media with approximately equal numbers of cells before and after harvest. We measured growth from the rate of increase of chlorophyll; although this method will not necessarily provide a measure of absolute growth rates, the existence of an abnormal lag phase will indicate if there are significant numbers of cells which are nonliving or whose growth rates have been rendered abnormal. Growth appeared to proceed normally except in the case of Syn, which showed a significant lag period (Fig. 3 a–d).

DISCUSSION

We have shown that several kinds of planktonic algae can be harvested from laboratory cultures by continuous-flow centrifugation into gradients of silica. The recovered cells are not seriously damaged in the process, as shown by their essentially normal rates of growth in fresh medium. The rates of sample flow are such that 50 liters of culture of the smallest algal cells could be harvested in a four-hour interval. The absolute quantities are limited only by the capacity of the gradient, which is of the order of tens of grams of cells.

Density gradient centrifugation is performed most often using sucrose as the gradient material. However, sucrose has the distinct disadvantage that the high osmotic potential of sucrose solutions will plasmolyze whole cells. Plasmolysis, in addition to the danger of working irreversible changes on the cells, has the effect of increasing the cell density. In fact, we found that none of our organisms could be floated in any concentration of sucrose-seawater up to 65% w/w nor in sorbitol-seawater up to 60% w/w.

Ficoll-seawater did provide a medium in which the cells could float and remain approximately normal in size and shape. We found that algae required up to 25%

w/w Ficoll in seawater, a solution that is extremely viscous and difficult to pump in and out of the rotors; in addition it is expensive.

Ludox, a silica sol, is non-osmotic like Ficoll, but poses neither the problem of viscosity nor expense. However, it has some peculiarities of its own: It has a tendency to gel in the presence of salts. We found that low concentrations of Ludox were stable in 3% w/v NaCl, but higher concentrations were not. We were obliged therefore to employ Ludox gradients in which the dense end of the gradient is deficient in salt with respect to ordinary seawater. For stenohaline organisms

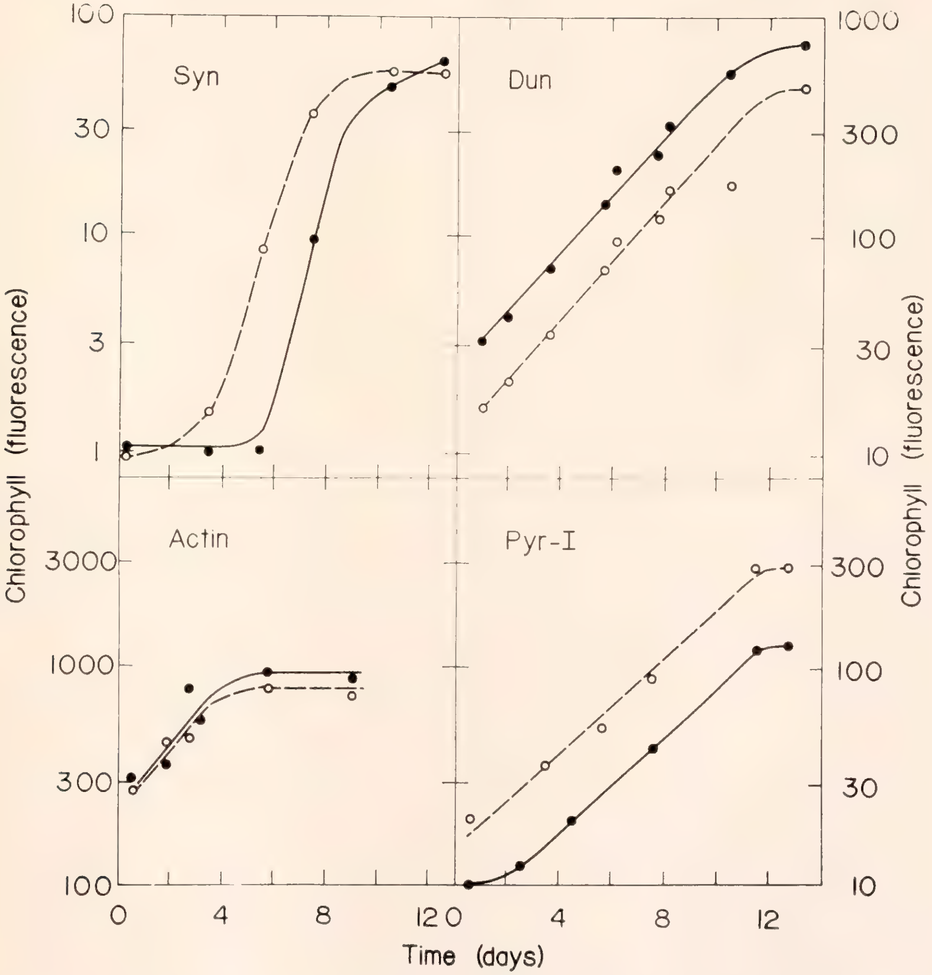

FIGURE 3. Comparison of growth rates of algae before and after harvest by centrifugation in Ludox gradients. Cell densities were estimated by the relative concentration of chlorophyll as determined by fluorescence. Similar numbers of cells were inoculated into fresh media at zero time. The absence of an abnormal lag period was taken as an indication of viability; *Synechococcus bacillaris* (Syn); *Dunaliella tertiolecta* (Dun); *Thalassiosira fluviatilis* (Actin); *Pyramimonas* sp. (Pyr-I).

the deficit in osmotic potential can be compensated with sorbitol or other compatible osmotica. We have also found some evidence of toxicity of Ludox.

The movements of flagellates, such as *Dunaliella*, ceased abruptly when exposed to even a low concentration of ordinary Ludox. We then found that this toxicity is removed by dialysis, but prolonged dialysis may substantially dilute the preparation. Silica sols free of added biocides have recently become available from Nalco Chemical Company, Chicago, Illinois. In some cases biological activity may be preserved by the addition of small amounts of polymers such as polyethylene glycol (Pertoft, 1969; Morgenthaler *et al.*, 1974).

The lowered rates of respiration and photosynthesis obtained after the organisms were harvested from Ludox gradients may, as in the case of Pyr-I, reflect short-term physiological effects, since growth appeared to proceed normally or after a brief lag period. Of the organisms tried here, Dun seemed to be the least affected by centrifugation in gradients of Ludox, whereas Syn manifested physiological damage, as shown by the increased lag period as well as decreased rates in respiration and photosynthesis. Since Dun occurs naturally in brackish waters, its insensitivity to Ludox gradients may be related to its tolerance of low concentrations of salt.

SUMMARY

1. Cells of four different phytoplankton species were harvested from cultures using gradients of colloidal silica (Ludox) in the CF-6, a continuous-flow zonal rotor.

2. The efficiency of harvesting ("fractional clean-out") was > 0.99 for three of the algae and > 0.78 for the smallest species (*Synechococcus bacillaris*).

3. Growth rates of the algae subcultured after harvesting in Ludox were similar to those unharvested controls, but *Synechococcus* showed a lag period of about four days after harvesting.

4. The rates of photosynthesis and respiration of cells harvested in Ludox gradients were, in most cases, about $\frac{1}{3}$ to $\frac{1}{5}$ of the pre-harvest rates. *Dunaliella tertiolecta* showed no loss of photosynthetic or respiratory activity.

5. This method has application to the collection of algae from dilute cultures and from natural waters when cells are needed in good physiological condition.

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GLUCOSE AND SODIUM FLUXES ACROSS THE BRUSH BORDER OF *HYMENOLEPIS DIMINUTA* (CESTODA)[1]

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Tapeworms are a favorable material for studying the transport of organic solutes across membranes. The outer surface of adult tapeworms is comprised of a coenocytic epithelium with a highly developed brush border (Lumsden, 1966). Since tapeworms have no digestive tract, nutrients from the external medium must cross this external tegument. In addition, the intact tapeworms can be easily and rapidly maniupulated, making them ideal subjects for the study of transport kinetics.

Previous studies have shown that glucose transport in several species of tapeworms is Na^+-dependent (von Brand and Gibbs, 1966; Fisher and Read, 1971; Dike and Read, 1971a; Pappas and Read, 1972a; Pappas, Uglem and Read, 1973a). The present study was undertaken to characterize in some detail the relationship of glucose and sodium fluxes in the rat tapeworm, *Hymenolepis diminuta.*

MATERIALS AND METHODS

Hymenolepis diminuta was maintained in the beetle, *Tenebrio molitor,* and male Sprague Dawley rats (Holtzman Co.) Rats weighing 60–80 g were infected with 30 cysticerocoids (*H. diminuta*) and worms were recovered 10 days post-infection. Worms from several rats were pooled and randomized into groups of 5, and preincubated for 15 min at 37 C in 15 ml of Krebs-Ringer saline containing 25 mM tris(hydroxymethyl)aminomethane-maleate buffer at pH 7.4 (KRT of Read, Rothman and Simmons, 1963), or in KRT with the NaCl replaced isomotically with KCl, LiCl, choline-Cl, or tris(hydroxymethyl)aminomethane-Cl. Other modifications of salines are described in the text. After preincubation worms were blotted on filter paper, placed in 5 ml of KRT (or the appropriate ion-substituted saline) containing radioactive substrate, and incubated for 2 min at 37 C. To terminate incubation each worm group was removed and rapidly rinsed in KRT, blotted on filter paper, and extracted overnight in 2 ml of 70% ethanol. Radioactivity in aliquots of the ethanol extracts and incubation media was determined using a gas-flow counter or liquid scintillation spectrometer; influx rates were calculated by comparing the radioactivity extracted from worms with the specific activity of the incubation media. The ethanol extracted worms were dried overnight at 95 C and weighed. Uniformly labeled ^{14}C-D-glucose and $^{22}Na^+$ (as NaCl) were obtained from Amersham/Searle Corp.

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[2] Deceased, December 24, 1973.

[3] N.I.H. Postdoctoral Fellow, 5-FO2-AI-45108–02. Present Address: Department of Zoology, The Ohio State University, Columbus, Ohio 43210.

RESULTS

Dike and Read (1971a) showed that the influx of glucose across the brush border of *H. diminuta* is sensitive to Na^+ in the incubation medium. Our preliminary experiments indicated that preincubation of worms in media without Na^+ also affects glucose influx. To examine this effect further, worms were preincubated for varying time periods in Na^+-free KRT with tris as the replacement cation, and then incubated for 2 min in Na^+-free KRT containing 1 mM ^{14}C-glucose. The results shown in Figure 1 demonstrated that a 12 min preincubation of worms in Na^+-free KRT was sufficient to inhibit glucose influx (J^i_G) 96%.

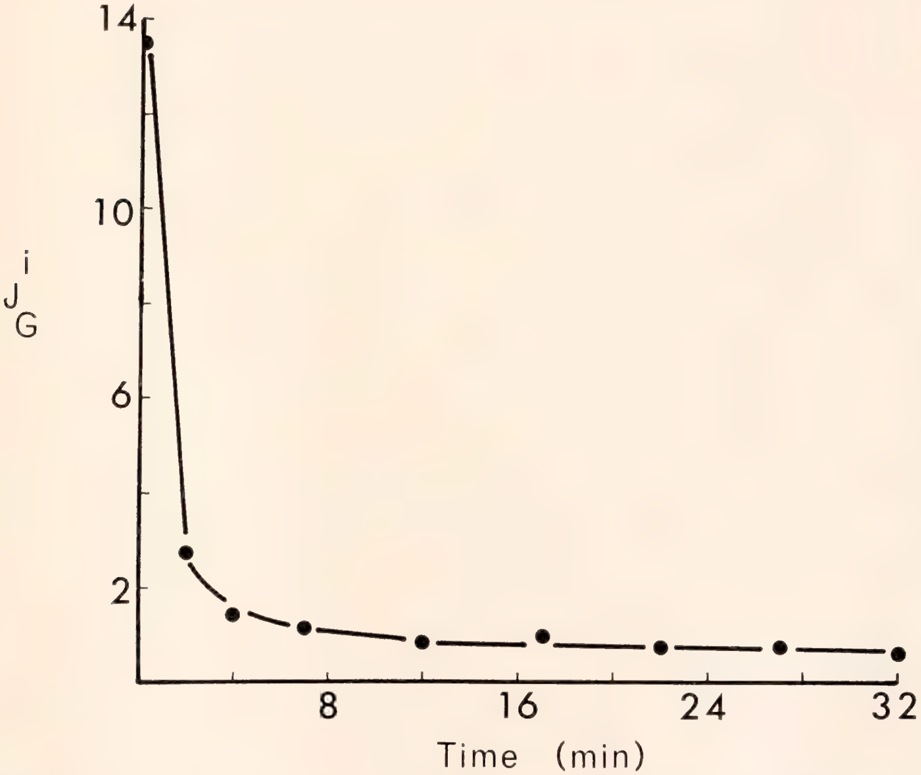

FIGURE 1. The effect of perincubation time in Na^+-free KRT on glucose influx (J^i_G = μmoles glucose absorbed/g ethanol extracted dry wt/2 min) in *Hymenolepis diminuta*. Worms were preincubated in Na^+-free KRT for predetermined time periods (abscissa), and then transferred to Na^+-free media containing 1 mM ^{14}C-glucose for 2 min. In this experiment, Na^+ was replaced with tris. Each point is the mean of 3 replicates.

To determine the reversibility of the effect of Na^+ deletion on glucose influx, worms were preincubated in Na^+-free media for 30 min, incubated in KRT for varying time periods, and subsequently incubated for 2 min in KRT containing 1 mM ^{14}C-glucose. The results shown in Figure 2 demonstrated complete reversal of the effect of Na^+ deletion. In all subsequent experiments, worms were pre-

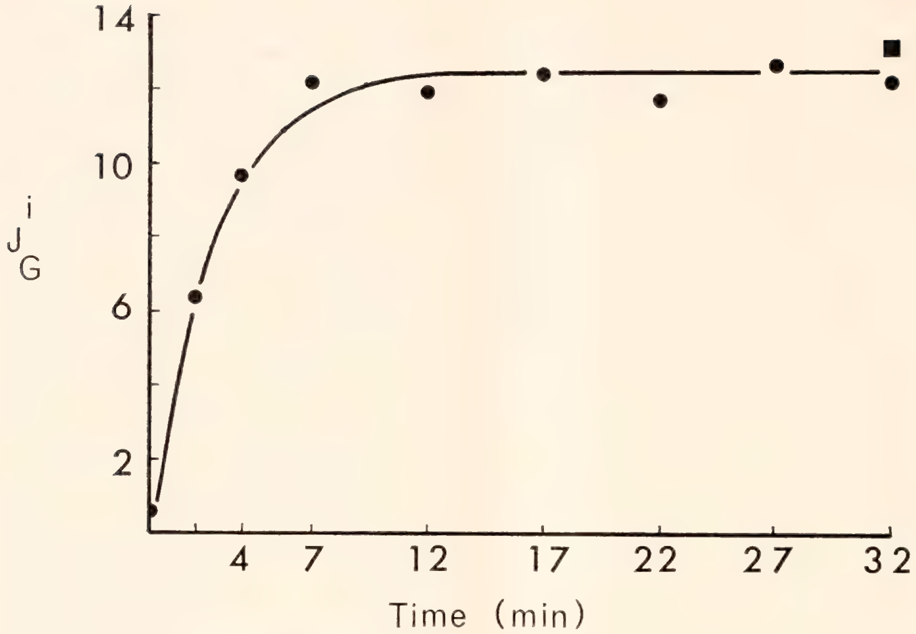

FIGURE 2. Reversal of the effects of Na$^+$ deletion on the influx of glucose (J^i_G, as in Fig. 1) in *Hymenolepis diminuta.* Worms were preincubated in Na$^+$-free KRT (tris as the replacement cation) for 30 min, followed by incubation in KRT ([Na$^+$] = 154 meq/l) for predetermined time periods (abscissa), and subsequently incubated in 1 mM ^{14}C-glucose in KRT for 2 min. The single square point represents glucose influx in a control group which was not incubated in Na$^+$-free KRT. Each point is the mean of 3 replicates.

incubated for 15 min in media containing the same cation concentrations in the final incubation medium.

It seemed desirable to examine the effects of various other cations as replacements for Na$^+$ in the medium. The effect on glucose influx of totally replacing Na$^+$ with Li$^+$, K$^+$, choline, or tris is shown in Table I. The much lowered influxes in media where K$^+$, tris, or choline were used as replacements for Na$^+$ were similar. However, glucose influx with Li$^+$ as the replacement cation was significantly higher ($P < 0.05$ by Student's t test) than influxes obtained with the other substituting

TABLE I

A comparison of 0.5 mM glucose influx ($J_G{}^i$ = μmoles glucose absorbed/g ethanol extracted dry wt/2 min) in Hymenolepis diminuta *in salines with the Na$^+$ totally replaced with various cations and KRT. Each value is the mean ± S.E. of 3 replicates*

Replacement Cation	$J_G{}^i$	$J_G{}^i$ in KRT
K$^+$	0.086 ± 0.005	5.66 ± 0.32
Choline	0.076 ± 0.006	4.96 ± 0.28
Tris	0.031 ± 0.004	5.28 ± 0.31
Li$^+$	0.56 ± 0.03	4.58 ± 0.22

cations, suggesting that Li^+ may partially replace Na^+. This "replacement" effect disappeared when increasing amounts of Na^+ were added to media containing Li^+. Glucose influx was a hyperbolic function of Na^+ concentration regardless of the replacement cation (Fig. 3).

Although the data did not indicate that K^+ competes with Na^+ in glucose influx, this possibility was examined further by determining the effects of K^+ concentrations on glucose influx at a constant glucose concentration (0.5 mM) and a suboptimal, fixed Na^+ concentration (25 meq/l) (isosmolarity was maintained by adding an appropriate amount of tris-Cl). There was no effect of K^+ on glucose influx at K^+ concentrations ranging from 0 to 100 meq/l.

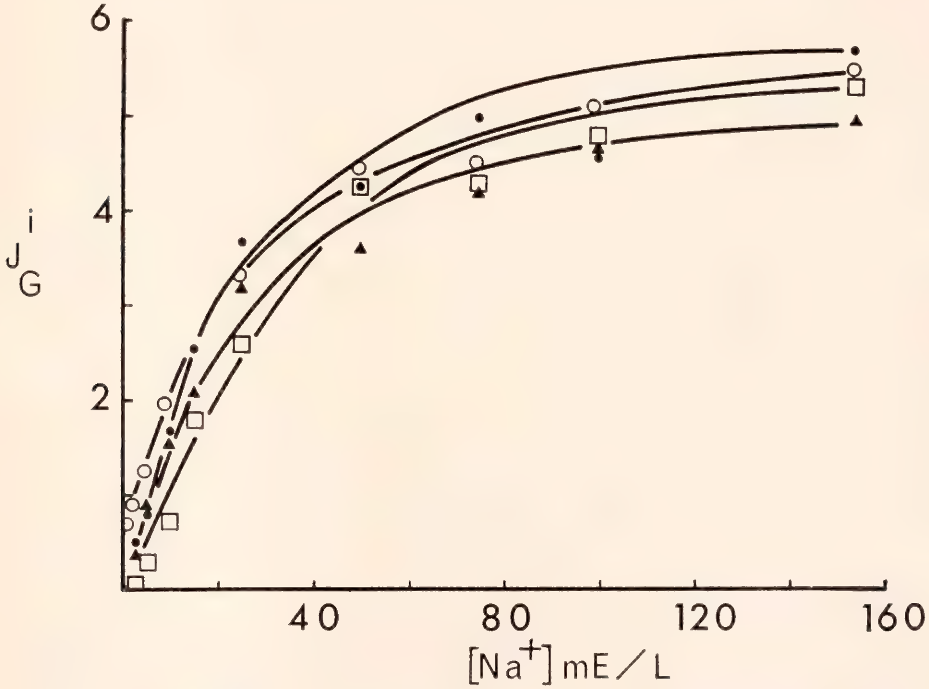

FIGURE 3. The influx of 0.5 mM ^{14}C-glucose (J^i_G as in Fig. 1) in *Hymenolepis diminuta* as a function of the Na^+ concentration ($[Na^+]$) in media with the Na^+ replaced with various cations. Replacement cations were K^+ (solid circles), Li^+ (open circles), tris (open squares), or choline (solid triangles). Each point is the mean of 3 replicates.

Glucose influx as a function of glucose concentration followed apparent Michaelis-Menten kinetics in media with fixed Na^+ concentrations of 154, 50, 25, or 10 meq/l (Fig. 4). The apparent transport constant (K_t) for glucose influx was affected only slightly with decreasing Na^+ concentrations, but there was a marked decrease in the maximal influx of glucose ($J^i_G{}^{max}$) with decreasing Na^+ concentration (Table II).

When glucose influx was tested as a function of Na^+ concentration at several fixed glucose concentrations, it was apparent that glucose influx was a hyperbolic function of Na^+ concentration, regardless of the glucose concentration. The data also showed that the Na^+ concentration necessary to achieve $J^i{}_G{}^{max}/2$ changed significantly at different glucose concentrations (Fig. 5, Table II). The kinetic parameters describing these glucose-Na^+ interactions are summarized in Table II.

While the above experiments demonstrated the dependence of glucose influx on Na^+, and suggested coupling of glucose and Na^+ fluxes, more direct evidence of coupling required a demonstration that Na^+ influx ($J^i{}_{Na}$) was related to the presence of glucose in the external medium. As shown in Table III, a very large Na^+ influx was associated with glucose influx.

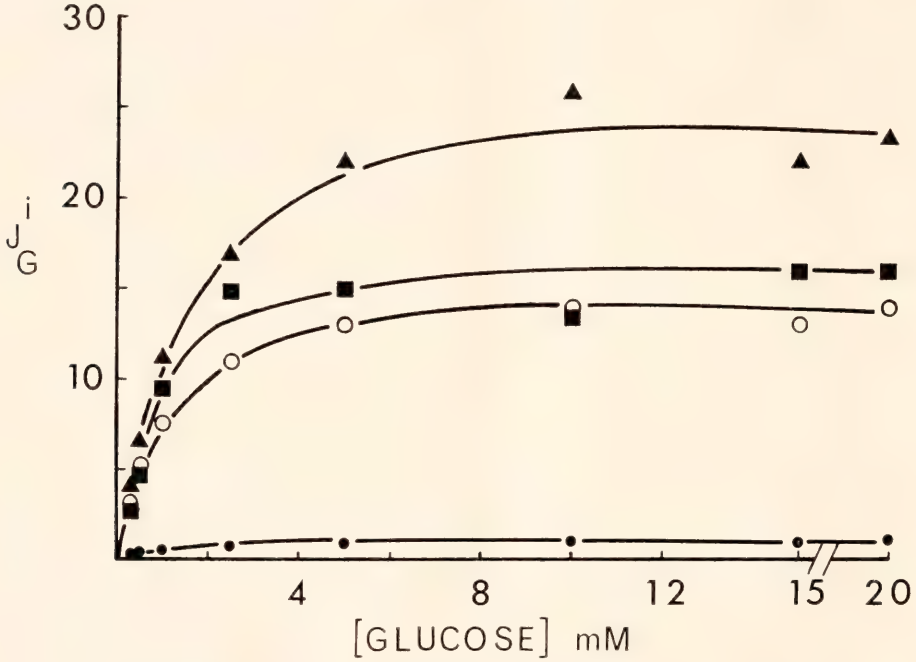

FIGURE 4. The influx of ^{14}C-glucose ($J^i{}_G$ as in Fig. 1) in *Hymenolepis diminuta* as a function of glucose concentration in media with different fixed Na^+ concentrations. Na^+ concentrations of 154 meq/l (solid triangles), 50 meq/l (solid squares), 25 meq/l (open circles), and 10 meq/l (solid circles) were used. In all experiments, deleted Na^+ was replaced with tris. Each point is the mean of 3 replicates.

Before carrying out experiments designed to evaluate coupling coefficients ($J^i{}_{Na}/J^i{}_G$), it was necessary to determine that the influxes of ^{14}C-glucose and $^{22}Na^+$ followed first order kinetics for the time period employed. Groups of worms were incubated in solutions containing either 25 meq/l $^{22}Na^+$ and 5 mM glucose, or 25 meq/l Na^+ and 5 mM ^{14}C-glucose. Samples were removed at 15 sec intervals, washed for approximately 2 sec in KRT and extracted in 70% ethanol. The influxes of Na^+ and glucose were a linear function of time for at least 2 min (Fig. 6).

To determine coupling cofficients, using labeled Na^+ and labeled glucose, incubations were conducted for 2 min with less than a 2 sec postincubation rinse in KRT.

The results of such an experiment are shown in Figure 7. In this experiment groups of worms were incubated in KRT containing 25 meq/l $^{22}Na^+$ and varying glucose concentrations, or 25 meq/l Na^+ with varying concentrations of ^{14}C-glucose. The plot of Na^+ influx versus glucose influx has a slope of 1.6 by regression analysis, indicating the coupling coefficient was at least 2. The intercept of the line in Figure 7 represents Na^+ influx not associated with glucose influx. As expected, the glucose-coupled Na^+ influx was a hyperbolic function of glucose concentration (Fig. 8). It should be noted that the concentration of Na^+ yielding a half-maximum rate of Na^+ influx in the presence of 5 mM glucose was about 154 meq/l. In a different type of experiment, the glucose concentration was held constant and the Na^+ concentration varied. Results of such an experiment are presented in Table IV. When $^{22}Na^+$ influx in the presence of glucose was corrected for the amount of Na^+ entering the worms in the absence of glucose, the apparent coupling coefficient varied as an inverse function of the Na^+ concentration. That is, with decreasing Na^+ concentrations the coupling coefficient increased. Thus, the coupling coefficient appeared independent of the glucose concentration over a 50-fold range, but inversely dependent on the Na^+ concentration over a 20-fold range.

TABLE II

A summary of the kinetic parameters describing ^{14}C-glucose influx in Hymenolepis diminuta *as functions of Na^+ and glucose concentrations (meq/l and mM, respectively). The graphic analysis of V vs. V/[S] (Dixon and Webb, 1964), with calculated regression lines, was used to determine these parameters*

Summary of Figure 4

$[Na^+]$	$J_G^{i\ max}$*	K_t**
154	26.3	1.4
50	17.1	1.0
25	14.7	0.9
10	1.1	0.8

Summary of Figure 5

[Glucose]	$J_G^{i\ max}$	K_t†
5	28.0	56
1	10.1	43
0.5	6.8	40
0.1	1.7	34

* maximal glucose influx = μmoles absorbed/g ethanol extracted dry wt/2 min.
** that [glucose] necessary to achieve ½ $J_G^{i\ max}$.
† that $[Na^+]$ necessary to achieve ½ $J_G^{i\ max}$.

When $^{22}Na^+$ influx was measured as a function of Na^+ concentration in the absence of glucose, it was found to be a nonlinear function of Na^+ concentration. This suggested that $^{22}Na^+$ influx in the absence of glucose did not occur by simple diffusion. This was examined further by adding varying quantities of unlabeled Na^+ in the presence of a fixed quantity of $^{22}Na^+$. If Na^+ influx was by diffusion alone, the addition of unlabeled Na^+ should not have affected the influx of $^{22}Na^+$.

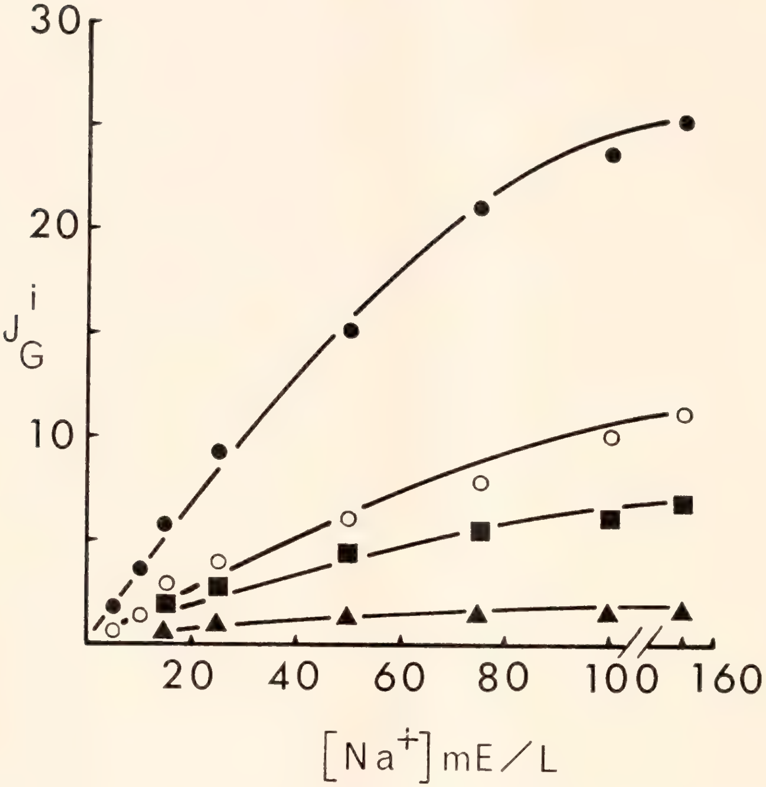

FIGURE 5. Influx of ^{14}C-glucose (J^i_G as in Fig. 1) in *Hymenolepis diminuta* as a function of Na$^+$ concentration in media with different glucose concentrations. Glucose concentrations of 5 mM (solid circles), 1 mM (open circles), 0.5 mM (solid squares), and 0.1 mM (solid triangles) were used. In all experiments, deleted Na$^+$ was replaced with tris. Each point is the mean of three replicates.

Such was not the case (Fig. 9). The data indicated that in the absence of glucose about 60% of the ^{22}Na$^+$ influx at this concentration (10 meq/l) occurred by a mediated process, and about 40% by diffusion.

TABLE III

Influx of ^{22}Na$^+$ (J_{Na}^i = µequivalents absorbed/g ethanol extracted dry wt/2 min) as a function of the Na$^+$ concentration ([Na$^+$], meq/l) in Hymenolepis diminuta *in the presence or absence of 5 mM glucose. Each value is the mean ± S.E. of 3 replicates*

[Na$^+$]	J_{Na}^i (with glucose)	J_{Na}^i (without glucose)
5	3.07 ± 0.28	0.7 ± 0.1
15	8.78 ± 0.51	0.9 ± 0.1
25	14.33 ± 1.21	1.38 ± 0.01
50	26.33 ± 2.11	1.64 ± 0.02
100	36.52 ± 2.93	3.22 ± 0.02

In the absence of external Na^{+}, glucose influx in *H. diminuta* as a function of glucose concentration appeared saturable, with an approximate $J^{i}_{G}{}^{max}$ and K_t of 0.15 μmoles/g ethanol extracted dry wt/2 min and 0.5 mM, respectively. The inhibition of 0.1 mM ^{14}C-glucose influx by unlabeled glucose indicated that at least 80% of the glucose influx in Na^{+}-free media was mediated.

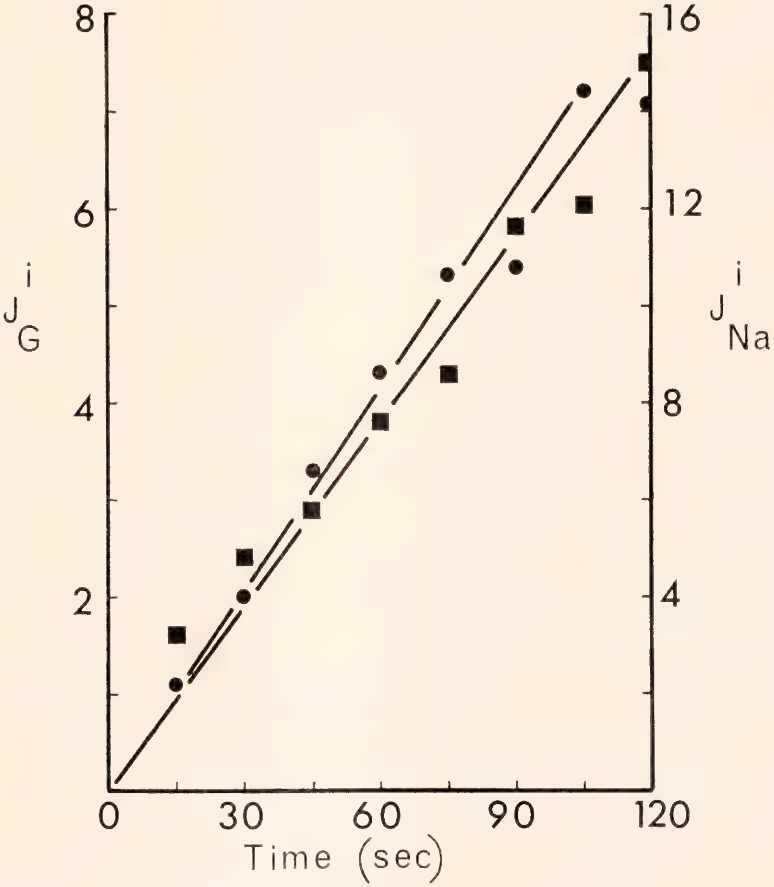

FIGURE 6. Fluxes of 5 mM ^{14}C-glucose (J^{i}_{G} = μmoles absorbed/g ethanol extracted dry wt, left ordinate) and 25 meq/l ^{22}Na (J^{i}_{Na} = μequivalents absorbed/g ethanol extracted dry wt, right ordinate) into *Hymenolepis diminuta* as a function of time; J^{i}_{G} = solid circles, J^{i}_{Na} = solid squares. Delected Na^{+} was replaced with tris. Each point is the mean of 3 replicates.

The above observations suggested that further studies of the mediated influx of ^{14}C-glucose in the absence of Na^{+}, and of the mediated portion of ^{22}Na^{+} movement in the absence of glucose, were desirable. The glycoside phlorizin was a competitive inhibitor of ^{14}C-glucose influx in *H. diminuta* (Fig. 10). In the absence of external Na^{+}, phlorizin abolished over 70% of the residual ^{14}-C- glucose influx suggesting that the same glucose transport system was involved in the presence and absence of external Na^{+}. On the other hand, phlorizin had no effect on the influx of $^{22}Na^+$ in the absence of glucose, suggesting the possibility of mediated Na^+ influx which was independent of the glucose transport system.

DISCUSSION

The data of the present paper are consistent with the basic elements of Crane's (1962, 1965) Na^+-gradient hypothesis for the transport and accumulation of sugars (see also Schultz and Curran, 1970). Previous studies (Pappas, Uglem and Read, 1974) have shown that glucose is indeed accumulated by *H. diminuta,* and cotransport of glucose and Na^+ across the brush border of *H. diminuta* is demonstrated unequivocally in the experiments described herein.

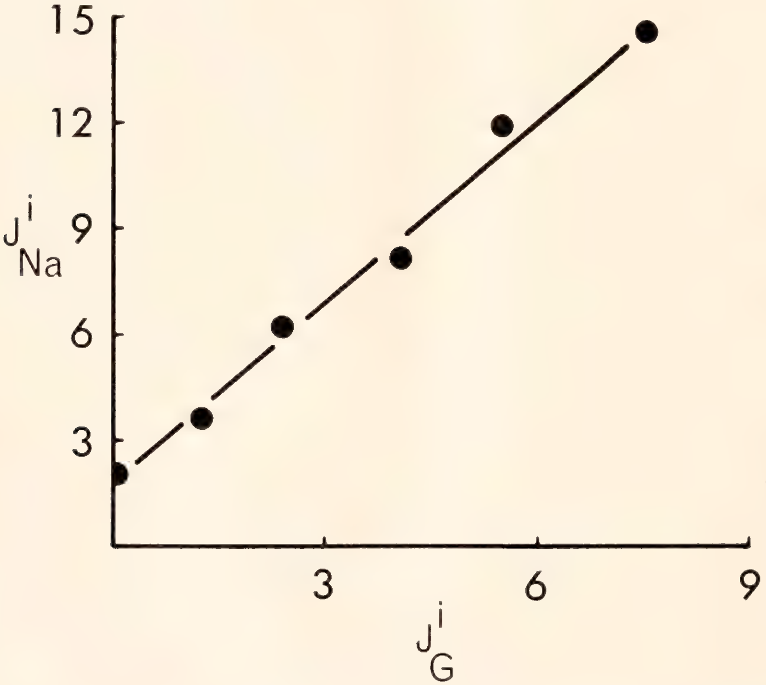

FIGURE 7. The relationship of ^{14}C-glucose influx (J^i_G as in Fig. 1) and $^{22}Na^+$ influx (J^i_{Na} = μequivalents absorbed/g ethanol extracted dry wt/2 min) in *Hymenolepis diminuta* when the Na^+ concentration was 25 meq/l, and the glucose concentration was 0, 0.1, 0.25, 0.5, 1 and 5 mM. Deleted Na^+ was replaced with tris. Each point is the mean of 3 replicates.

When Na^+ is deleted from the medium, K^+, choline, or tris do not substitute for Na^+ in enhancing glucose influx, while Li^+ appears to substitute to a small extent. The effects of Li^+ disappear with increasing Na^+ concentrations. Similar effects of Li^+ on glucose influx in the tapeworm *Calliobothrium verticillatum* were reported by Pappas and Read (1972a). Pappas *et al.* (1973a) found that K^+ competes with Na^+ in the glucose transport system of the larval tapeworm *Taenia crassiceps,* and a similar interaction between K^+ and Na^+ has been observed in the tetra-

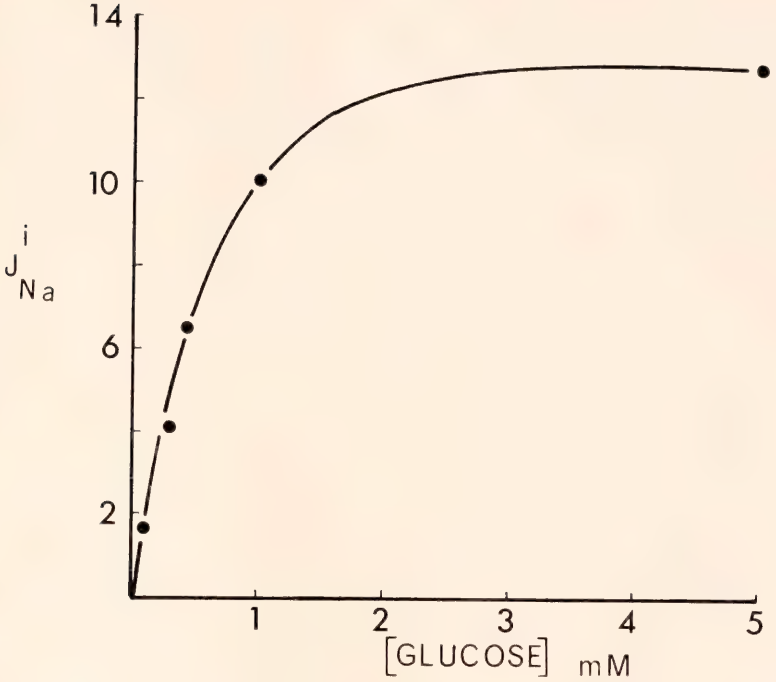

FIGURE 8. Influx of 25 meq/l $^{22}Na^+$ (J^i_{Na} as in Fig. 7) in *Hymenolepis diminuta* as a function of glucose concentration. All data were corrected for that Na^+ movement which occurs in the absence of glucose. Each point is the mean of 3 replicates, and deleted Na^+ was replaced with tris.

phyllidean *C. verticillatum* by Fisher and Read (1971) and Pappas and Read (1972a). Competition between Na^+ and K^+ in Na^+-dependent transport systems in mammalian tissues is suggested by the data of Nathans, Tapley and Ross (1960), Kipnis and Parrish (1965), and Crane (1965). In contrast to the above systems, K^+ does not appear to interact with the Na^+-coupled glucose transport system of *H. diminuta*. Further, there is no evidence that glucose transport in *H. diminuta* is inhibited in K^+-free media, as has been observed in some mammalian tissues (Riklis and Quastel, 1958; Bihler and Crane, 1962). When the external Na^+

TABLE IV

The coupling coefficient (J_{Na}^i/J_G^i) *for* Na^+*-coupled glucose influx in* Hymenolepis diminuta *as a function of* Na^+ *concentration of the external medium* ([Na^+], *meq/l*). *In each experiment the* Na^+ *concentration was held constant and the glucose concentration varied from 0.1 to 5* mM. *Coupling coefficients are calculated slopes of plots of* J_{Na}^i vs. J_G^i, *as in Figure 7*

N*	[Na^+]	Coupling coefficient
18	5	1.90
18	25	1.60
18	50	0.87

* number of determinations used in calculating each coupling coefficient.

concentration is greater than zero, glucose influx in *H. diminuta* conforms to apparent Michaelis-Menten kinetics, and a decreased external Na^+ concentration results in a lower maximal glucose influx, as well as a somewhat lower K_t. Thus, between 10 and 154 meq/l Na^+, $J^i{}_G{}^{max}$ changes about 25-fold, while K_t changes less than 2-fold. This difference indicates that high Na^+ concentrations decrease slightly the affinity of the carrier for glucose, but that the major effect of increasing Na^+ on glucose influx is an increased translocation rate of the complex formed by the carrier, glucose, and Na^+.

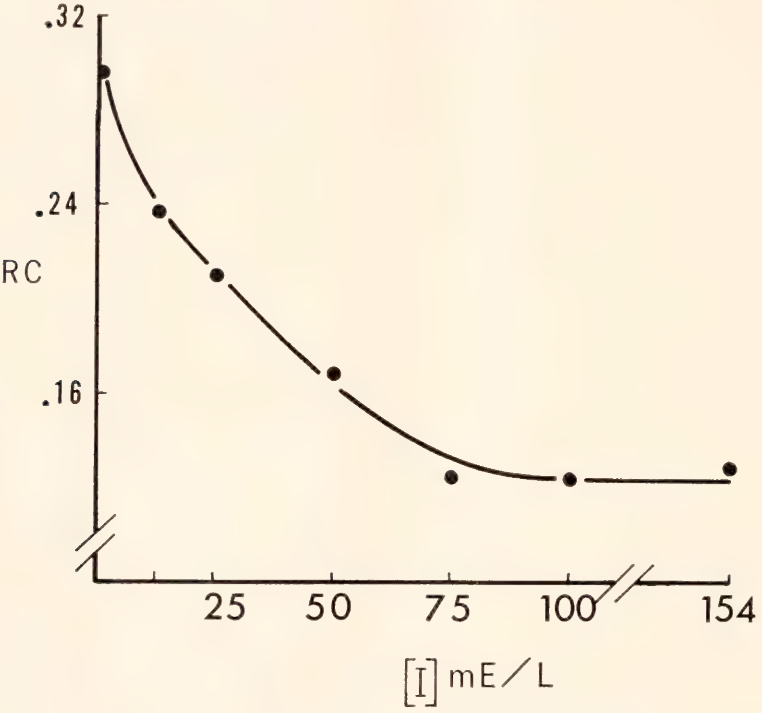

FIGURE 9. A plot of the rate constant (RC) for the influx of 10 meq/l $^{22}Na^+$ in *Hymenolepis diminuta* as a function of increasing concentrations of unlabeled Na^+ in the medium ([I]). Deleted Na^+ was replaced with tris, and appropriate amounts of tris were added such that $[Na^+] + [tris] = 154$ meq/l. The rate constant was calculated as follows: ([μequivalents of $^{22}Na^+$ absorbed by *H. diminuta*/ml worm water/2 min]/[μequivalents $^{22}Na^+$/ml incubation medium]). Thus, the units of the rate constrant are $(time)^{-1}$.

As previously reported by Dike and Read (1971a), the influx of glucose shows first order dependence on the Na^+ concentration of the medium. In this respect, *H. diminuta* resembles *C. verticillatum* (Pappas and Read, 1972a), and the mucosa of rabbit ileum (Goldner, Schultz and Curran, 1969). However, in *H. diminuta,* the coupling coefficient ($J^i{}_{Na}/J^i{}_G$) varies inversely with the Na^+ concentration, whereas in *C. verticillatum* and in the rabbit iluem the coupling coefficient is independent of the Na^+ concentration. In *C. verticillatum*, the coupling coefficient is 2 or more, and in rabbit iluem it is unity. The coupling coefficients appear to be independent of the glucose concentration in all of these systems.

In showing first order dependence on Na⁺ concentration with coupling coefficients greater than unity, glucose influx in *H. diminuta* resembles Na⁺-coupled glucose influx in *C. verticillatum* (Pappas and Read, 1972a), and alanine influx in the pigeon erythrocyte (Wheeler and Christensen, 1967). In the latter system, Na⁺-coupled influxes of asparagine, hydroxyproline, serine, and threonine have coupling coefficients of 2 or more, but show a first order dependence on the Na⁺ concentration in the suspending medium (Koser and Christensen, 1968). On the other hand, *H. diminuta* differs from the above mentioned systems in that the coupling coefficient is an inverse function of the Na⁺ concentration.

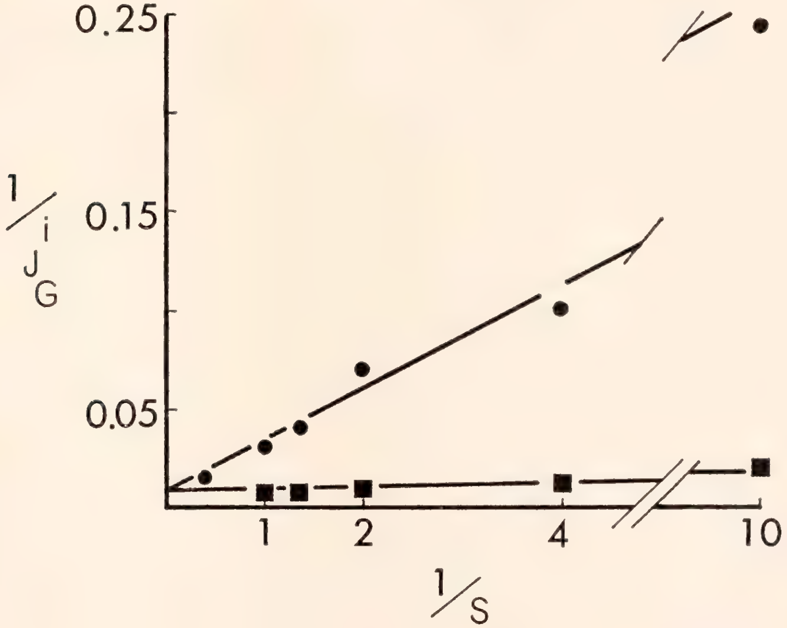

FIGURE 10. Effect of 0.05 mM phlorizin on the 2 min influx of ^{14}C-glucose (J^i_G as in Fig. 1) in *Hymenolepis diminuta;* S = glucose concentration, mM; influxes with phlorizin = solid circles; influxes without phlorizin = solid squares. Each point is the mean of 3 replicates, and lines were fitted by regression analysis.

Any kinetic model which will describe the coupled influx of Na⁺ and glucose across the brush border of *H. diminuta* must account for all of the properties of the *H. diminuta* system indicated above, particularly the first order dependence on Na⁺ concentration with coupling coefficients exceeding unity. Such a model must include the following: (1) The binary complex of carrier and glucose, or binary or higher complexes of carrier and Na⁺ (one or more Na⁺), are translocated to a very minor extent. (2) Binding of one Na⁺ to the carrier at a single specific site results in translocation of the glucose-carrier-Na⁺ tertiary complex. (3) If the glucose-carrier complex is translocated at a very low rate, although free carrier is freely translocated, there should be a tendency for pooling of this complex in the *cis* configuration (*i.e.*, at the external surface of the membrane) at very low Na^+ concentrations with glucose in the medium. (4) Further, binding of Na^+ apparently can occur in at least one non-effector site on the carrier, with only one site involved in effecting the translocation of the glucose-carrier-Na^+ complex; a change in the Na^+ concentration in the medium may change the coupling coefficient if the binding constants of the noneffector and effector Na^+ sites differ considerably.

Pappas and Read (1972a) observed coupling coefficients of at least 2 in the cotransport of glucose and Na^+ in *C. verticillatum*. It was postulated that there are 2 or more Na^+ binding sites on the glucose carrier, that there is no cooperative interaction between Na^+ binding sites, and that binding of Na^+ on any site will effect translocation of the glucose-carrier complex. However, *H. diminuta* differs from *C. verticillatum* in that the coupling coefficient changes as a function of Na^+ concentration, and the model postulated for *C. verticillatum* will not apply to *H. diminuta*.

With the above in mind, the following hypothesis will furnish a tentative explanation of the data: The glucose carrier has multiple sites for the binding of Na^+. Na^+ binding on at least one of these sites has negliglibe effector function in the translocation of glucose, and the non-effector site(s) is saturated at very low Na^+ concentrations. Translocation of Na^+ bound to non-effector sites is glucose-dependent, although the translocation of glucose is not dependent on Na^+ bound to non-effector sites. Binding of Na^+ at a single effector site acts in the translocation of glucose, and this effector site is not saturated at physiological concentations of Na^+. Thus, in the presence of glucose and low external Na^+ concentrations, binding and translocation of Na^+ on low affinity effector and high affinity non-effector sites would yield coupling coefficients of 2 or more. As the Na^+ increases to concentrations well above those which saturate the non-effector site, the Na^+ cotransported after combining with the non-saturated effector site becomes a larger and larger proportion of the cotransported Na^+, and the coupling coefficient decreases, approaching unity. This hypothesis may be amenable to experimental testing.

A small mediated influx of glucose occurs in the absence of Na^+ in the medium with *H. diminuta*, and Pappas and Read (1972a) found a small, but significant, mediated transport of glucose in the absence of Na^+ in *C. verticillatum*. However, in neither case can it be concluded that this involves a truly Na^+-free environment for the carrier. In *H. diminuta* there appears to be an unstirred layer adjacent to the glucose transport system which is not freely accessible to proteins (glucose oxidase) (Dike and Read, 1971a). Since Na^+ may well leak into this unstirred layer, the residual mediated transport of glucose, without Na^+ in the external medium, might involve the coupled transport of Na^+ and glucose, the latter of which has leaked into the unstirred layer from the worm's tissues. However, the failure of phlorizin to inhibit Na^+ influx in the absence of glucose argues against this latter hypothesis. It is of interest to note that in larvae of *T. crassiceps*, glucose influx in the absence of Na^+ occurs by diffusion (Pappas *et al.*, 1973a). Attempts to determine coupling coefficients for the influx of Na^+ and glucose in *T. crassiceps* have failed, although mediated glucose influx is Na^+-dependent. Influx of Na^+ in the absence of glucose is extremely high in *T. crassiceps* larvae, and coupled Na^+ influx may be masked (Pappas and Uglem, unpublished; Pappas *et al.*, 1973a).

On the other hand, measured coupling coefficients may be zero, though coupling occurs, if the rate constants for the processes involved are appropriate (Schultz and Curran, 1970). An elucidation of the nature of glucose and Na^+ movements in *T. crassiceps* larvae requires further experimentation.

Crane's (1965) data on the transport of sugar and Na^+ in the hamster intestine indicated that there is cooperative interaction between the binding sites. There is negligible cooperative interaction in binding of sugar and Na^+ in *H. diminuta*, and there is no evidence for such an interaction in *C. verticillatum* or rabbit mucosa (Pappas and Read, 1972a; Goldner *et al.*, 1969). In the two tapeworms and in rabbit mucosa, the main effect of Na^+ on glucose tansport seems to involve translocation of the glucose-carrier complex, rather than an effect of Na^+ on the affinity of the carrier for glucose.

H. diminuta shows a remarkable array of variations in dependence of transport on Na^+. Glucose transport is Na^+-dependent and Na^+-coupled. Glycerol transport is partially Na^+-dependent (Pittman and Fisher, 1972), and it has been shown that there are possibly two glycerol systems involved, one of which is Na^+-dependent and inhibited by 1,2-propanediol, and another which is insensitive to Na^+ and 1,2-propanediol. However, coupling of Na^+ and glycerol fluxes was not demonstratble (Uglem, Pappas and Read, 1974). In addition to *H. diminuta*, there is evidence for a relationship between Na^+ and sugar transport in three other tapeworms species, *Taenia taeniaeformis, T. crassiceps*, and *C. verticillatum* (von Brand and Gibbs, 1966; Fisher and Read, 1971; Pappas *et al.*, 1973a; Pappas and Read, 1972a). Contrary to the Na^+-dependence of glucose fluxes in *H. diminuta* and *T. crassiceps* larvae, amino acid fluxes and accumulation in these two tapeworms are apparently Na^+-insensitive (Read *et al.*, 1963; Pappas, Uglem and Read, 1973b, 1974).

The coupling of Na^+ and glucose fluxes in parasitic flatworms is of some interest from an evolutionary standpoint. It is generally agreed that flatworms constitute a primitive group of multicellular organisms. The occurrence of coupled Na^+ and sugar fluxes in such disparate systems as the hamster and rabbit brush borders and absorptive surfaces of tapeworms furnishes a strong argument that such systems are widely distributed in the animal kingdom, and that the evolution of such systems has been very conservative. Further, although it can be argued that obligately parasitic tapeworms have lost regulatory capacities in evolution (Read and Simmons, 1963), *H. diminuta* has retained a complex array of independent systems for regulating fluxes of organic solutes across the brush border. Specific transport systems in *H. diminuta* include at least four for amino acids (Read *et al.*, 1963), one for sugars (Phifer, 1960; Read, 1961), two for glycerol (Pittman and Fisher, 1972; Uglem *et al.*, 1974), at least three systems for purine and pyrimidine bases (MacInnis, Fisher and Read, 1965; MacInnis and Ridley, 1969; Pappas, Uglem and Read, 1973c), one for short-chain fatty acids (Arme and Read, 1968), one for long-chain fatty acids (Chappell, Arme and Read, 1969), and at least two for water-soluble vitamins (Pappas and Read, 1972b 1972c). In addition, *H. diminuta* possesses intrinsic tegumentary phosphohydrolases (Arme and Read, 1970; Dike and Read, 1971a, 1971b; Pappas and Read, 1974) which, in conjunction with the above transport systems, may play an important role in regulating organic fluxes across the tegument. Further, *H. diminuta* may also alter the kinetic properties of host enzymes (Pappas and Read, 1972d, 1972e; Read, 1973; Ruff and Read, 1973).

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SUMMARY

When *Hymenolepis diminuta* was preincubated in Na^+-free KRT (tris-maleate buffered Krebs-Ringer saline) for varying time intervals, followed by incubation in ^{14}C-glucose in Na^+-fee KRT, the influx of glucose in worms was lowered significantly. This effect of Na^+ deletion on glucose influx was totally reversible by incubating worms in KRT ($[Na^+] = 154$ meq/l).

When Na^+ in the medium was replaced with K^+, tris, or choline, a similar decrease in glucose influx in worms was noted; replacement of Na^+ with Li^+ resulted in a glucose influx rate significantly higher than that obtained with K^+, tris, or choline as the replacement cation. In media with a suboptimal Na^+ concentration (25 meq/l), influx of 0.5 mM glucose in worms was unaffected by varying concentrations of K^+ (0–100 meq/l).

Apparent Michaelis-Menten kinetics were observed when glucose influx as a function of glucose concentration was determined in media with Na^+ concentrations of 154, 50, 25, and 10 meq/l. There was a slight decrease in the apparent transport constant (K_t) for glucose influx and a marked decrease in maximal glucose influx ($J^i{}_G{}^{max}$) with decreasing Na^+ concentration. Glucose influx in *H. diminuta* as a function of the Na^+ concentration displayed apparent Michaelis-Menten kinetics, and the Na^+ concentration necessary to attain $J^i{}_G{}^{max}/2$ lowered significantly at lower glucose concentrations.

A large Na^+ influx was associated with glucose influx in *H. diminuta*, and the influxes of Na^+ and glucose exhibited first order kinetics for at least 2 min. In media with Na^+ concentrations of 5, 25, and 50 meq/l, coupling coefficients ($J^i{}_{Na}/J^i{}_G$) were 1.90, 1.60, and 0.87, respectively, or inversely related to the Na^+ concentration. Coupling coefficients were independent of the glucose concentration over a 50-fold range. Glucose-coupled Na^+ influx in worms was a hyperbolic function of glucose concentration. In the absence of glucose, Na^+ influx in *H. diminuta* apparently occurred, in part, by a mediated process which was unaffected by phlorizin. There was a small mediated glucose influx in worms in the absence of external Na^+, 70% of which was abolished by phlorizin.

The cotransport of Na^+ and glucose in *H. diminuta* is (1) compared with other similar systems in both tapeworms and mammals, (2) presented in support of Crane's Na^+-gradient hypothesis for transport and accumulation of sugars, (3) tentatively explained in the form of a hypothesized model, and (4) discussed briefly in relation to the evolution of parasitic flatworms.

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NATURE AND ROLE OF PROTEINACEOUS HORMONAL FACTORS ACTING DURING PUPARIUM FORMATION IN FLIES

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In 1969, Zdarek and Fraenkel found that hemolymph from early puparia or neurosecretory material from the pars intercerebralis of the brain would greatly accelerate puparium formation in flies. After further investigation of the puparium-accelerating reactions from the CNS and the hemolymph, Fraenkel, Zdarek and Sivasubramanian (1972) proposed two alternative models for the neuroendocrine events that occur during pupariation in flies. The first implied that ecdysone stimulates the synthesis and/or storage and release into the hemolymph of two proteinaceous factors, one leading to retraction of the anterior segments into the body (X_r), and the second causing tanning (X_t). A different neurosecretory product from the central nervous system (CNS) accelerates the release of these proteinaceous factors. The second model was based on an assumption of the identity of the acceleration factors in the CNS and hemolymph. According to this a neurosecetory product from the brain is stored in peripheral nerves or at nerve endings before the critical period, and ultimately released into the hemolymph as the X-factors by the action of ecdysone. Injections of brain extracts supply the X-factors into the blood sooner and thus accelerate pupariation.

The schemes suggested by Fraenkel *et al.* (1972) raised several questions. Are there really two distinct protein factors controlling retraction of the anterior segments and tanning? How are the hemolymph factors related to the brain factors? Do they originate from the brain or are they synthesized in some other tissue(s)? If the X-factors do exist, to what extent can they replace ecdysone during pupariation? The subject of this investigation is to seek answers to these questions and to clarify the existence, nature and action of the X-factors during pupariation in the larva of the fleshfly, *Sarcophaga bullata* Parker. A fuller presentation of this work is given elsewhere (Sivasubramanian, 1973).

In the present study X_r (the retraction factor) and X_t (the tanning factor) will be referred to, respectively, as ARF (anterior (segments) retraction factor) and PTF (puparium tanning factor). Since the bioassay for the factors depended on the acceleration of the respective processes they were originally called acceleration factors by Fraenkel *et al.* (1972). However, all available evidence points to the fact that retraction and tanning cannot occur in the absence of these factors. Hence they will be referred to in this paper as hormonal factors instead of acceleration factors.

MATERIALS AND METHODS

Experimental animal

The insect used throughout this study was *Sarcophaga bullata*. Staging the larvae for use as hosts and donors in bioassay: *Hosts*, about 3–4 hours before pu-

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pariation of the larva of *Sarcophaga bullata* the cuticle between and around the posterior spiracles begins to tan. This is a clear indication of forthcoming pupariation and a good time to obtain larvae of uniform age. Larvae of this stage, called red-spiracle larvae, were found to be most responsive as hosts for experiments on pupariation (Zdarek and Fraenkel, 1969) and, unless otherwise stated, have been used as hosts in this study. *Donors*, according to Fraenkel *et al.* (1972), the hemolymph from the orange puparium of *Sarcophaga bullata* (about 1 hour after the formation of the white puparium) is highly active with respect to the X-factors that are involved in immobilization of larvae (during puparium formation) by retraction of anterior segments and tanning. Therefore, orange puparia were used as donors of hemolymph. In the same report it was shown that the pupariation-accelerating activity of the CNS changes very little from the mature larva (crop-emptied, postfeeding stage about 10–12 hours before pupariation) until 16 hours after puparium formation. Therefore, mature larvae were used as CNS donors.

Method of injection

Host larvae were immobilized on ice and injected according to the techniques of Zdarek and Fraenkel (1969), using glass pipettes with a tip diameter of about 40 μ.

Ligation techniques

Ligatures with heavy duty cotton thread were applied well behind the brain-ring gland complex (approximately on the 6th segment) in such a way that the neural and endocrine sources were isolated anterior to the ligation.

Bioassay methods

Intact and ligated host larvae were used in the bioassays for ARF and PTF. The activities of both factors were determined in intact larvae but only tanning could be scored in ligated larvae.

Scoring the results

After injection the larvae were left on filter paper in Petri dishes, and examined every 10 minutes. In intact larvae the time taken for the retraction of the anterior segments and that taken for tanning were expressed as percentages of controls injected with *Drosophila* Ringer solution. In the ligated red spiracle larva there is already enough ecdysone to induce tanning on both sides of the ligature, but the anterior part always tans before the posterior part because the CNS in the former liberates certain pupariation acceleration factors (Zdarek and Fraenkel, 1969). Thus, in the Ringer-injected controls, the quotient of the pretanning period in minutes of the posterior part over the anterior part (P/A) is always more than 1. If the hind parts are injected with material containing the hormonal factor they tan before the anterior part and the quotient of P/A is less than one.

In order to express activities quantitatively, increasing amounts of protein with ARF and PTF activities were injected into intact and ligated hind parts of red-spiracle larvae, respectively. The time taken for retraction of the anterior segments under the influence of ARF, expressed in terms of a percentage of control time, was plotted as a function of the amount of protein injected. Similarly, the time taken for tanning after PTF injection, as expressed by the P/A ratio of the ligated larva was plotted as a function of the quantity of protein injected. Each curve was linear over a portion of its range, so the quantity of protein necessary to effect either retraction of anterior segments or tanning at the mid point of the linear part of the curve was considered as 1 unit of activity. This was at 50% of control time for ARF and at a P/A ratio of 1 for PTF.

Protein was determined according to the method of Lowry, Rosebrough, Farr and Randall (1951), using crystalline bovine serum albumin (BSA) as a standard.

Isolation of active factors

All procedures involved in the fractionation of hemolymph and CNS were carried out at 2° to 4° C. In the collection of hemolymph and CNS, the hemolymph was expressed from cut anterior ends of orange puparia into a centrifuge tube containing a few crystals of phenylthiourea. In order to dissect the CNS, mature larvae were immobilized on ice and their mouth hooks plus the first three body segments chopped off. A gentle pressure on the body brought out the CNS with a little bit of fat body. This was teased away in Ringer solution and the CNS were preserved in acetone and stored at 0–4° C until use. Then the acetone was decanted and the dry CNS's were homogenized in Ringer solution at a concentration of 100 CNS per ml and centrifuged in the cold (5° C) at 105,000 *g* for 50 minutes in a Beckman Model L-2 ultracentrifuge. The supernatant (cytosol) was used for further fractionation.

Enzyme hydrolysis. Approximately 10 mg of "Enzite-Try" (Immobilized trypsin, Miles-Seravac (PTY) Ltd, Maidenhead, Berks, England) was added to 1 ml of the sample and the mixture incubated at 37° C for 1–2 hours. After incubation the enzyme was sedimented by centrifugation at 2000 *g* for 10 minutes and the supernatant tested for activity. The sample was incubated with an equal volume of 0.1% pepsin in 0.01 N HCl for 2 hours at 37° C. Then the enzyme was inactivated by heating to 100° C for 3 minutes.

Ultrafiltration. Ultrafiltration of soluble fractions was performed with Amicon permselective membranes (Amicon Corp., Lexington, Massachusetts 02173) graded in terms of molecular weight cut off levels.

Gel filtration. Bio-Gel P-300 (100–150 mesh) (Bio-Rad Labs., Richmond, California 94804) was hydrated with Ringer solution and packed in a column of 1.6 × 36 cm size using the method suggested by the manufacturers. About 2 ml of sample, consisting of a total of about 250 mg of protein, was applied on the column and eluted with Ringer solution. One-ml fractions were collected at a rate of 3 ml per hour and tested for activity as previously described. Bovine liver catalase (Sigma), beef heart LDH (Sigma) and *Escherichia coli* alkaline phosphatase (Worthington) were used as markers for molecular weight determinations.

Gel electrophoresis. Seven per cent (W/V) polyacrylamide gels were prepared in gel tubes 9 × 0.7 cm, and disc electrophoresis carried out using the techniques outlined by Davis (1964). Current during the run was 3 mAmp per tube. Active fractions of hemolymph (from Bio-Gel P-300 column) or CNS (cytosol fraction retained by XM-300 filter) equivalent to about 500 μg protein were used. After electrophoresis gels were fixed for 30 minutes in 12.5% trichloroacetic acid and stained with Coomassie brilliant blue solution (one volume of 1% aqueous CBB solution plus 19 volumes of 12.5% TCA). Gels were destained in 10% TCA and stored in 7.5% acetic acid.

For preparative electrophoresis the method was exactly the same as that described for analytical electrophoresis except that at the end of a run the gels were neither fixed nor stained. Instead they were sliced and the proteins extracted with Ringer solution overnight in the cold. After concentration by filtering through the Amicon membranes the supernatants were used for assay.

The technique of electroelution used was that of Maizel (1964) with certain chamber modifications. Electrophoresis through 7% polyacrylamide was employed to separate proteins, but was continued until each protein had completely traversed the gel, and moved out into a small elution chamber at the bottom of the gel tube. Buffer was continuously pumped through the chamber and the fractions were collected separately using a fraction collector.

Estimation of molecular weights. The molecular weights of pure active samples were determined by SDS (sodium dodecyl sulfate)-gel electrophoresis using a modification of the method of Weber and Osborn (1969).

Five volumes of the sample or a standard (yeast alcohol dehydrogenase, bovine liver catalase and bovine serum albumin, all from Sigma Chemical Corp. St. Louis, Missouri, and proteins of potato yellow dwarf virus with known molecular weights obtained from Dr. R. McLeod, Department of Botany, University of Illinois, Urbana), were mixed with 1 volume of 0.05 M sodium phosphate buffer containing 10% SDS, 10% mercaptoethanol and 1 mg of urea and boiled for 5 minutes. They were then prepared for the run as follows: Protein solution after boiling, 10 μl (about 10 μg); 0.01 M phosphate buffer containing 1% SDS and 1% mercaptoethanol, 25 μl; 0.05% bromophenol blue in water, 5 μl; Mercaptoethanol, 5 μl; 40% sucrose in water, 2 drops. The above solutions were mixed and run on a 7 × 0.6 cm column of 7% acrylamide containing 0.1% SDS and 8 M urea at 6 mAmp per tube at room temperature for about 4 hours. The gels were stained for 2 hours in 0.25% Coomassie brilliant blue in methanol, water and glacial acetic acid (5:5:1). After staining the gels were kept for at least 30 minutes in a destaining solution consisting of glacial acetic acid, methanol and water (3:2:35) and then destained electrophoretically for 2 hours in the same solution.

RESULTS

In all the following experiments the volume of sample injected per host was 8 μl. The desired amounts of proteins to be injected were made up to 8 μl with Ringer solution and then injected.

Unfractionated sample

Hemolymph from orange puparia was collected and centrifuged in the cold for 10 minutes at 700 *g*. Injection of the supernatant plasma into intact red-spiracle larvae indicated that at least 105 μg of hemolymph protein are required to effect retraction of anterior segments in 50% of control time (1 ARF unit). About 3 times more protein had to be injected to observe PTF activity in ligated larvae (Table I).

TABLE I

Effect of injection of various quantities of hemolymph from orange puparia into intact and ligated hind parts of red-spiracle larvae of S. bullata. *(One ml of hemolymph contains about 205 mg of protein.) The figures are means from 20 observations*

Quantity of hemolymph protein injected (μg)	Retraction % Control*	Tanning	
		% Control**	P/A***
0 (Ringer sol.)	100	100	1.33 ± 0.03
820	24.5 ± 0.74	55.2 ± 2.3	0.73 ± 0.034
410	23.7 ± 0.86	64.8 ± 5.1	0.79 ± 0.036
300	25.6 ± 0.67	65.9 ± 2.8	1.05 ± 0.006
205	27.0 ± 0.93	69.4 ± 5.8	1.18 ± 0.047
105	52.6 ± 1.4	76.6 ± 3.6	1.29 ± 0.02
80	75.1 ± 2.3	89.0 ± 4.2	1.26 ± 0.005
55	>100	>100	1.33 ± 0.06

* Time required for retraction of anterior segments after injection as % of time required for Ringer's-injected control.

** Time required for tanning of whole larvae after injection as % of time required for Ringer's-injected control.

*** Quotient of the pretanning period of the posterior part of ligated larvae over that of the anterior part. The same notations are used in the subsequent tables.

The cytosol fraction of the CNS was injected into intact and ligated hind parts of red-spiracle larvae (Table II). Injection of 10 μg of cytosol protein per host accelerated tanning to such an extent that intact hosts were still crawling when tanning had already set in. The effect of this quantity of cytosol on retraction was relatively small. Thus, it appears that CNS extract has a greater effect on tanning than on retraction of the anterior segments whereas the opposite is true of hemolymph.

Precipitation of active factors with ammonium sulfate

The hemolymph proteins precipitated with various concentrations of ammonium sulfate were assayed for ARF and PTF activities. The 45–70% ammonium

TABLE II

Effect of injection of various quantities of cytosol fraction of CNS homogenate into red-spiracle larvae of S. bullata

Quantity of cytosol protein of CNS injected (μg)	Retraction % Control	Tanning	
		Control	P/A
0 (Ringer sol.)	100	100	1.28 ± 0.01
10	86 ± 5.0	74 ± 3.6	0.73 ± 0.05
5	95 ± 2.1	79 ± 4.1	0.80 ± 0.0
3	>100	85 ± 1.61	0.91 ± 0.06
2	>100	>100	1.02 ± 0.03
1.5	>100	>100	1.10 ± 0.005
0.75	>100	>100	1.18 ± 0.02

sulfate fraction retained about 75% of the activity of both factors and, most importantly, did not darken and subsequently become toxic to the test animals, so this material was used for further fractionation. Ninety μg of protein from a 45–70% ammonium sulfate fraction had to be injected to get ARF activity equivalent to 1 unit, and 4 times more protein had to be injected to get a similar amount of PTF activity.

Ammonium sulfate precipitation of the cytosol fraction of the CNS was unsuccessful and very little PTF and ARF activities were retained in a 0–70% ammonium sulfate precipitate. Therefore, attempts to precipiate the activity were discontinued, and the original cytosol fraction was used for further fractionations.

Ultrafiltration

The 45–70% ammonium sulfate fraction of hemolymph was filtered through Amicon permselective membranes and tested for activity. The ARF activity passed through an XM-300 filter but was retained by an XM-100 filter indicating that the molecular weight of ARF was between 100,000 and 300,000. The fraction retained by XM-300 showed all of the tanning (PTF) activity.

The cytosol fraction of the CNS was filtered through Amicon filters and the fractions retained by the filters were assayed for activity. The fraction retained by an XM-300 filter (molecular weight above 300,000) retained all the PTF activity, and also showed slight ARF activity when higher concentrations of the fraction were injected.

Taken together the ultrafiltration experiments indicated that the gross molecular weight of ARF of hemolymph is between 100,000 and 300,000; and that of PTF from hemolymph and CNS is above 300,000.

Heat treatment

The 45–75% ammonium sulfate fraction of the hemolymph from orange puparia was dialyzed overnight and diluted 10 times with Ringer solution. Aliquots were then heated for 3 minutes at 60°, 80° and 100° C, then cooled and centrifuged at 10,000 *g* for 10 minutes. At 60° C there was no precipitation but the sample turned milky. At 80° C there was slight precipitation and at 100° C most of the proteins had precipitated. After centrifugation the supernatants were concentrated by filtering through an XM-50 filter and asasyed for ARF and PTF activities. The results indicated that ARF was destroyed by heating at 100° C whereas PTF was not. Heating at 100° C for 60 minutes still gave considerable PTF activity upon injection of 5 μg per larva.

Since the ultrafiltration studies showed that the fraction retained by the XM-300 filter had tanning activity and heating experiments revealed that PTF was not destroyed by heat treatment, the question arose as to whether heating had any effect on the size of the molecules which were originally quite large. As revealed in Table III, a high PTF activity is present in the unheated material as well as in the supernatant of the preparation heated to 100° C. In the unheated material PTF activity was present only in the fraction retained by XM-300 whereas after heating to 100° C almost all the activity was present in the fraction which passed through XM-100 but was retained by the XM-50 ultrafilter.

TABLE III

Comparison of the effect of injection of the active ammonium sulfate (45–70%) fraction of hemolymph before and after heat-treatment at 100° C for 3 minutes. The fractions were filtered through Amicon ultrafilters before assaying for tanning activity. The figures in parentheses are quantities of protein (μg) injected per host. The figures are means from 20 observations

Molecular weight of the fraction	P/A*	
	Unheated, 45–70% fraction	Supernatant of heated 45–70% fraction
Unfractionated	0.86 (600)	0.71 (30)
>300,000 daltons	0.88 (60)	1.0 (30)
100,000 to 300,000 daltons	1.00 (60)	1.16 (30)
50,000 to 100,000 daltons	1.21 (60)	0.76 (30)
Less than 50,000 daltons	1.19 (60)	1.22 (30)

* Ringer injected controls had a P/A ratio of 1.26.

In order to see whether the tanning activity is stable to heating, aliquots of the cytosol fraction of the CNS homogenate were heated for 3 minutes at 60° C, 80° C and 100° C. The fractions turned milky but there was no precipitation. The entire milky solution was assayed for PTF activity and the results clearly demonstrated that tanning activity was not lost after heating to 100° C for 3 minutes. There also remained an apparent, albeit very slight acceleration of retraction of the anterior segments after heating to 100° C. This will be taken up in some later experiments (Table IV).

To find out whether heating broke down the cytosol PTF molecules, the heat treated fractions were filtered through XM-300 ultrafilters and their protein content determined. There was no change in the protein content of the fraction retained by XM-300.

Enzyme hydrolysis

Treatment of the 45–70% ammonium sulfate fraction of hemolymph with trypsin and pepsin for 1–2 hours at 37° C revealed that both ARF and PTF are inactivated by these proteolytic enzymes.

Fraenkel *et al.* (1972) reported that PTF activity of CNS was somewhat resistant to pepsin. This was investigated in greater detail with the XM-300

TABLE IV

Effect of injection of heat-treated cytosol fractions of CNS into intact and ligated hind parts of red-spiracle larvae of S. bullata

Injected fractions	Quantity of protein injected (μg)	Retraction % control	Tanning P/A
Ringer solution	0	100	1.3 ± 0.05
Cytosol, unheated	15	76 ± 2.6	0.66 ± 0.02
Cytosol, heated to 60° C for 3 min.	15	74 ± 3.2	0.73 ± 0.02
Cytosol, heated to 80° C for 3 min.	15	91 ± 2.9	0.70 ± 0.09
Cytosol, heated to 100° C for 3 min.	15	93 ± 1.6	0.79 ± 0.00

retained fraction of CNS cytosol, and the previous observation confirmed. However, ultrafiltration of the pepsin treated fraction through XM-300 membranes revealed that digestion was incomplete over the time of the experiment, and that about half of the material with a molecular weight of above 300,000 had not been cleaved. Hence the apparent PTF activity after enzyme activity could be due to the presence of undigested molecules.

These data on the effect of proteolytic enzymes indicate that both ARF and PTF activities are destroyed after enzyme hydrolysis, which is one more evidence of their protein nature.

Gel filtration of hemolymph fraction

Since the results of the ultrafiltration experiments indicated that the approximate molecular weight range of the ARF active material was between 100,000 and 300,000, Bio-Gel P-300 was chosen for gel filtration. The 45–70% ammonium sulfate fraction was redissolved in Ringer solution and about 250 mg protein applied to a column with a bed volume of about 75 ml. One milliliter fractions were collected at a rate of 3 ml per hour.

The protein content of each fraction after the void volume (27 ml) was determined and every third sample was tested for ARF activity by injecting a volume equivalent to 15 μg of protein into each red-spiracle larva. The fractions eluted between 41 ml and 47 ml showed high ARF and tanning activity. These were pooled, concentrated by filtering through an XM-100 filter and then used for electrophoresis. From a plot of molecular weight as a function of elution volume using standard proteins with known molecular weights such as catalase (240,000), lactic dehydrogenase (140,000) and alkaline phosphatase (80,000) (Fig. 1) the molecular weight range of the active fractions was found to be between 180,000 and 220,000.

Electrophoresis

About 500 μg of hemolymph protein from the ARF active fractions after gel filtration was layered on each gel tube and electrophoresed till the marker dye reached about 0.5 cm from the bottom of the gel. The gels were then fixed, stained, and examined. No difference could be seen between whole hemolymph and gel filtration fractions with regard to the number and position of bands. In each case there were about 13 bands (Fig. 2A & B).

To examine PTF activity, the 45–70% ammonium sulfate precipitate was heated to 100° C for 3 minutes, the supernatant concentrated by filtering through an XM-50 ultrafilter and the material remaining above the filter electrophoresed. There were only four major bands in the gel (Fig. 2C).

The cytosol fraction of the CNS and the fraction that was retained by the XM-300 filter were electrophoresed by the same procedure. In both cases the bands were very faint. There were about 11 bands in the whole cytosol fraction and only 5 in the fraction retained by the XM-300 filter (Fig. 2D & E). The pattern was obviously different from that of the hemolymph fraction.

In order to extract the hemolymph proteins from the gel, 7 gels were run simultaneously using the ARF active fractions of gel filtration and at the end of electrophoresis the gels were removed from the tubes and one of them was fixed and stained in Coomassie brilliant blue. The other six gels were sliced into two unequal lengths, the upper (cathodal side, slice #II of 4 cm in length), and lower (anodal side, slice #I of 3 cm in length), and the slices pooled. Elution and testing of the proteins from these pooled fractions showed that the lower part of the gel, *i.e.*, slice #I, was more active than the upper part (Table V).

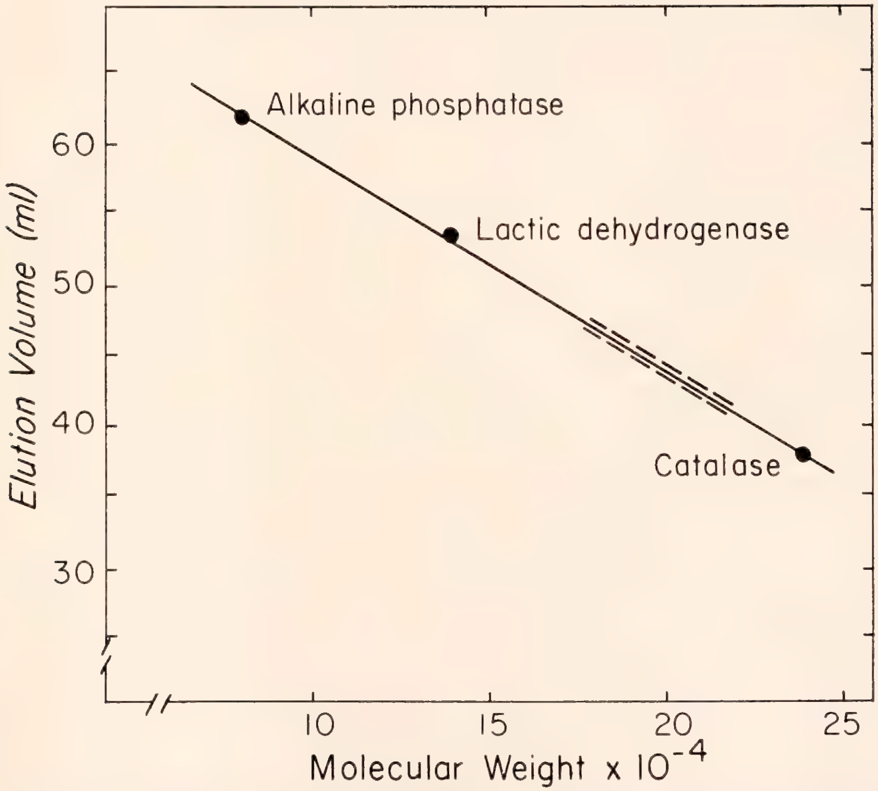

FIGURE 1. Molecular weight determination based on the flow rate through Bio-Gel P-300 column. Total bed volume was 75 ml. Flow rate was 3 ml per hour. The area in dotted line represents the region where the hemolymph fractions were active for ARF.

Knowing that ARF activity was present in the lower part of the gel, other gels were run and sliced into 14 equal parts of 0.5 cm length each (the total length of the gel was usually about 7 cm). Each slice was crushed into smaller bits in 1 ml Ringer solution, extracted overnight at 0–4° C, and the eluants tested as usual. The slices were numbered 1–14 beginning from the anodal end. As shown in Table V a relatively high ARF activity was seen in the extracts of slice #5.

The next step was to electroelute the proteins directly from the gel. Since the approximate location of the activity was known (slice #5, Table V), gels were electrophoresed for various periods of time after the dye front emigrated out of the gel and examined by staining procedures. It was observed that by about

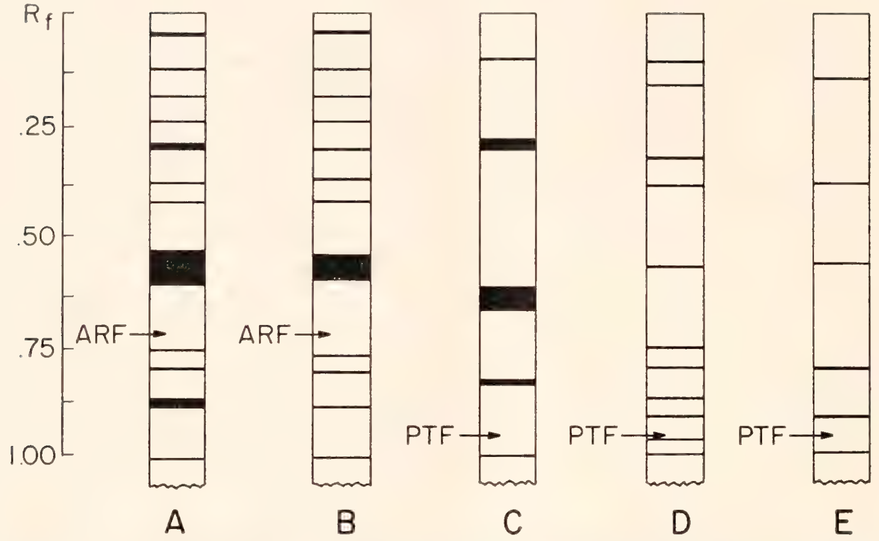

FIGURE 2. Electrophoresis of active fractions from hemolymph and CNS on 7% acrylamide gels. Arrows indicate the active regions; A., Whole hemolymph; B., Bio-gel P-300 active fraction of hemolymph; C., PTF-active supernatant of heat treated ammonium sulfate (45–70%) fractions of hemolymph; D., Cytosol fraction of CNS; E., Cytosol fraction of CNS retained by XM-300 filter.

TABLE V

Effect of injection of extracts obtained from electrophoresis of an active ARF fraction of hemolymph The results have been averaged from 20 observations. The hemolymph was processed in a P-300 column and the active fractions used for gel electrophoresis

Gel slice number*	Amount of protein injected (μg)	% Control time	
		Retraction	Tanning
I (Lower)	10	40.5	66
II (Upper)	10	75.6	82
1	3.5	100	100
2	5	100	100
3	5	100	100
4	13.5	88	91
5	5.5	81	79
6	5.5	95	94
7	12.5	95	97
8	6.5	100	100
9	6.0	96	92
10	3.5	96	94
11	5.5	90	89
12	7.0	100	100
13	9.0	100	100
14	7.0	100	100

* See text.

TABLE VI

Summary of purification steps of anterior segments retraction factor (ARF) from one ml of hemolymph (retraction in 50% of control time equals one Sarcophaga *unit for ARF).*

Fractions	Total protein (mg)	Total number of ARF units	Specific activity (units mg)	Purification fold	Yield (%)
Hemolymph	205	1990	9.7	1	100
45–70% ammonium sulfate precipitate	125	1388	11.1	1.13	69.7
Bio-Gel P-300 fractions	20	1333	66.6	6.86	67
Electro-eluted fractions	0.56	933	1666	171.7	46.9

3½ hours after the start the active region had migrated to the lowest part of the gel. Therefore, collection of fractions was begun about 3¼ hours after the start of electrophoresis. Ten fractions, of 0.7 ml volume, were collected at intervals of 5 minutes and each was assayed for ARF activity. Fraction #3 collected exactly 3½ hours after the start of electrophoresis seemed to be most active. However, injection of even 6 μg (10 units) of this protein did not accelerate tanning in the ligated hosts, indicating a complete absence of PTF.

A summary of the purification steps of the hemolymph retraction factor is given in Table VI. It has been purified about 170 times. The PTF containing fraction of hemolymph (both the supernatant of 100° C-heated 45–70% ammonium sulfate fraction, and the unheated 45–70% ammonium sulfate fraction retained by the XM-300 filter) were electrophoresed and the gels sliced into 6 equal parts. The proteins were eluted and the concentrated eluants were assayed for PTF activity. In both cases, the eluant from the gel slice closest to the dye front contained the highest PTF activity.

As shown in Table VII, the PTF has been purified 200 times. Since there is no gain in purification by ammonium sulfate precipitation it might be advisable in future to omit this procedure and to prevent darkening of the hemolymph by the same heating step used for purification.

About 300 μg of XM-300 retained protein from the CNS cytosol fraction was electrophoresed in 7% acrylamide gels. The gels were sliced and the proteins eluted, re-concentrated by filtering through XM-300 and, finally, tested for activity. The results showed that the eluant from the slice closest to the dye front contained

TABLE VII

Summary of purification steps of puparium tanning factor (PTF) of 1 ml of hemolymph. A P/A ratio of 1 equals 1 Sarcophaga *unit for PTF*

Fraction	Total protein (mg)	Total number of PTF units	Specific activity	Purification fold	Yield (%)
Hemolymph	205	685	3.3	1	100
45–70% ammonium sulfate precipitate	125	348	2.8	—	51
Supernatant of 100° C heat treated ammonium sulfate precipitate	1.65	330	200	61	48
Eluted from electrophoresed gel slice	0.3	200	667	202	29.2

the PTF activity and injection of 0.6 μg of this protein gave a P/A ratio of 1. The entire series of steps, beginning from the cytosol fraction of the CNS, increased the purity of PTF 5 fold (Table VIII).

The electrophoretic runs of the active fractions from hemolymph and the CNS have shown that ARF and PTF are distinct entities which have different mobilities in gels run under conditions specified above. ARF is located in the middle third of the gel, and PTF in the lowest part of the gel.

Estimation of molecular weight of the active fractions

The molecular weight of the most active electroeluted ARF fraction of hemolymph was determined by SDS-gel electrophoresis. Five separate runs of the sample gave a single band with an average mobility of 0.37. From the semi-log plot of marker proteins and their mobilities (Fig. 3) the molecular weight of ARF was estimated to be about 90,000. Bio-gel P-300 column chromatography showed the molecular weight of the active sample to be between 180,000 and 220,000 (Fig. 1). Since the subunit molecular weight appears to be about 90,000, the molecular weight of the native protein with high ARF activity is probably about 180,000 with 2 subunits of 90,000 each.

TABLE VIII

Summary of purification steps of puparium tanning factor from 100 CNS of mature larvae of Sarcophaga bullata *P*

Fraction	Total protein (mg)	Total number of PTF units	Specific activity	Purification fold	Yield (%)
Cytosol	1.71	570	333	1	100
Cytosol retained by XM-300	0.690	460	667	2	80.6
Eluted from the gel slice	0.120	200	1665	5	35

By the same procedure, the mobility of the gel-slice eluant of hemolymph having high PTF activity was found to be 0.74, which means a molecular weight of the subunit of PTF of about 26,000. Since the native protein had a molecular weight of more than 300,000, the molecule must have at least 12 subunits with a molecular weight of 26,000 each. (Fig. 3).

The active PTF fraction of the CNS eluted from the gel slice gave a single band with an average mobility of 0.41. From a semi-log plot of the marker proteins and their mobilities (Fig. 3) the molecular weight of PTF was estimated to be about 80,000. Since the native protein had a molecular weight of more than 300,000, this PTF fraction contains at least 4 subunits with a molecular weight of 80,000 each.

Molecular weight determinations by SDS-gel electrophoresis gave different mobilities (Fig. 3) and subunit molecular weights for ARF and PTF. The subunit molecular weight of ARF of hemolymph is about 90,000. The PTF from the CNS has a subunit molecular weight of about 80,000 and that from the hemolymph about 26,000.

Is there ARF activity in the CNS?

The ease of demonstrating PTF activity in the CNS is accentuated by the difficulty of showing ARF activity. In fact, only very high concentrations of the CNS fractions (cytosol and the fraction retained by XM-300 filter) injected into intact

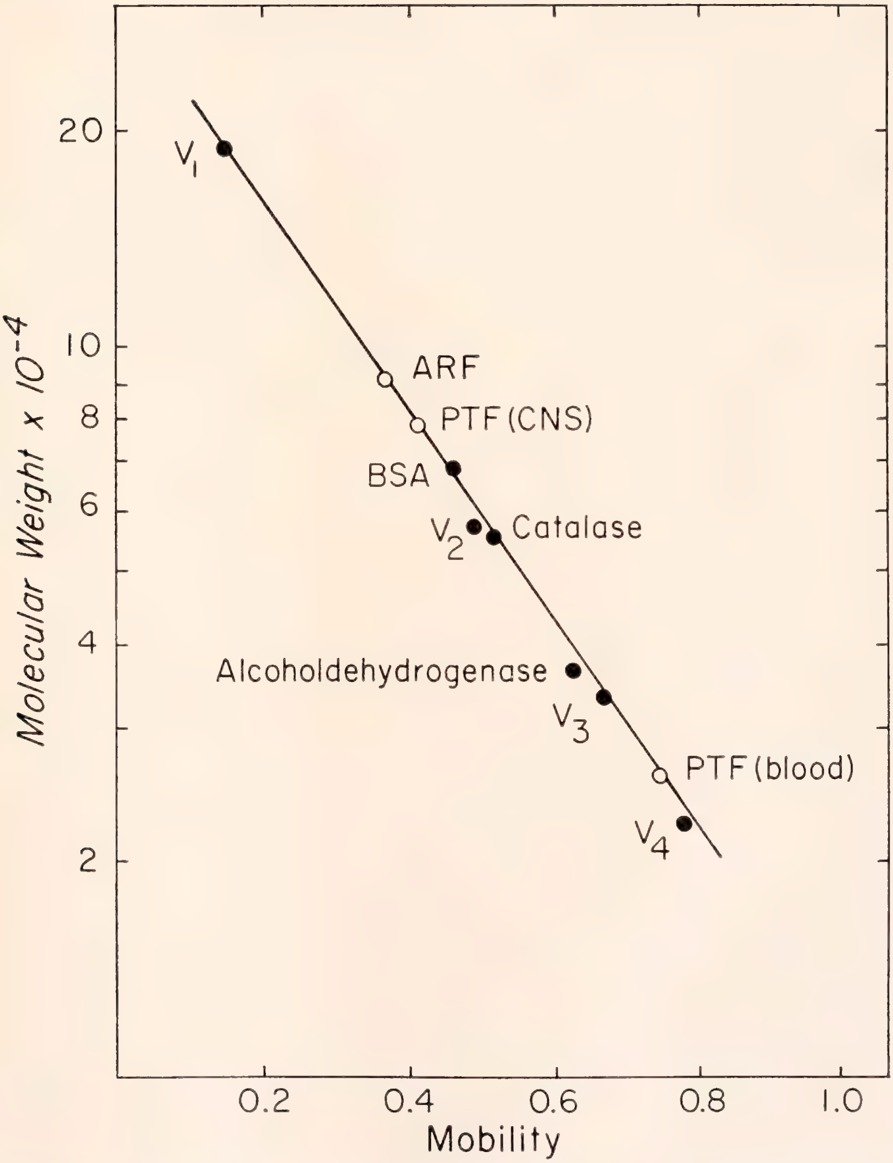

FIGURE 3. Semilog plot of molecular weight as a function of mobility of marker proteins using SDS-gel electrophoresis; V_1–V_4: Proteins of potato yellow dwarf virus with known molecular weights; ARF: Anterior retraction factor; PTF: Puparium tanning factor; BSA Bovine serum albumin.

red-spiracle larvae result in slightly accerelated anterior segment retraction (Table II). There are two possible explanations for this. Either the fractions do contain ARF in low concentration or, alternatively, the highly accelerated tanning and hardening of the cuticle might physically somehow make the hosts withdraw their mouth hooks before controls do. In order to clarify this point the above two fractions were injected with or without α-MDH into intact red-spiracle larvae of *Sarcophaga bullata*. α-MDH ((DL)-α-Methyl-α-hydrazino-β-(3,4-dihydroxyphenyl) propionic acid monohydrate) is a potent DOPA decarboxylase inhibitor and injection of 1 mμ-mole of this material into adult fleshflies has been shown to inhibit tanning almost completely (Seligman, Friedman and Fraenkel, 1969). As shown in Table IX, the cytosol fraction had ARF activity even when the tanning process was inhibited and this activity was lost after heating at 100° C for 3 minutes. However, the fraction retained by the XM-300 filter caused no acceleration of mouth hook retraction, when injected with α-MDH. Therefore the accelerated mouth hook retraction that occurs after injection of this fraction is probably not due to the presence of ARF but is due to some physical process that forces the larvae to withdraw their mouth hooks when the tanning process has been greatly accelerated.

TABLE IX

Effect of injection of the CNS fractions with or without α-MDH into red-spiracle larvae of S. bullata. *The figures are means from 20 observations. The quantities injected per host larva were, CNS fraction: 10 μg; MDH: 1 mμ-mole*

Fraction	% Control	
	Retraction	Tanning
Ringer	100	100
α-MDH	100	*
Cytosol	70	68
Cytosol plus α-MDH	81	*
Cytosol heated to 100°C plus α-MDH	100	*
Cytosol retained by XM-300 filter	83	79
Cytosol retained by XM-300 filter plus α-MDH	100	*

* No tanning takes place in presence of α-MDH.

The results of this experiment also indicate that the ARF which is present in the whole cytosol fraction probably has a MW below 300 K, since no activity remains after filtration through an XM-300 filter.

Can the hormonal factors (ARF and PTF) replace ecdysone?

In order to see whether or not the hormonal factors can replace ecdysone in whole or in part with regard to pupariation, several active fractions were injected into larvae in 3 different stages either with or without simultaneous injection of β-ecdyosne. Since 0.018 μg of β-ecdysone per larva was reported to be the critical dose for *Sarcophaga peregrina* (Ohtaki, Milkman and Williams, 1967), a subthreshold dose of 0.01 μg and a suprathreshold dose of 0.3 μg of β-ecdysone were injected. The subthreshold dose was used on the assumption that the hormonal factors might need some ecdysone in order to enable them to penetrate the target tissues.

The fractions were injected at two different periods: either simultaneously with ecdysone or an hour after ecdysone injection. The purpose of injecting an hour after ecdysone was to give the fractions an opportunity to act if they, like ecdysone, have a short half life, but can only function after ecdysone has exerted its action.

The samples injected included (a) 8 μl of active hemolymph from orange puparia (which is equivalent to 15 ARF units and 5 PTF units), (b) five μg each (equivalent to about 10 units each) of ARF and PTF from the fractions eluted from electrophoresed gels, or (c) ten μg (equivalent to 5 PTF units) of cytosol fraction of the CNS.

Three different stages of host larvae used for injections were (i) pre-critical stage larvae, about 36 hours before pupariation which contain no or very little ecdysone, (ii) early post-critical stage larvae, about 12 hours before pupariation which contain some ecdysone and (iii) X-irradiated (10,000 R) pre-critical stage larvae. In the X-irradiated larvae, the synthesis of β-ecdysone is temporarily blocked (Sivasubramanian, Ducoff and Fraenkel, 1974) and hence such larvae are suitable to test the activity of the hormonal factors in the absence of ecdysone.

The results of this experiment were completely negative. Neither the hormonal factors from the hemolymph nor the CNS induced puparium formation. They accelerated puparium formation, as seen in all the previous experiments, only in a red-spiracle stage larva, *i.e.*, long after ecdysone has triggered the initial events of pupariation.

DISCUSSION

In a previous report two alternative models for the hormonal events that occur during puparium formation were proposed (Fraenkel *et al.*, 1972). One of these assumed that the synthesis of "X-factors" which were responsible for the acceleration of puparium formation and tanning was induced by ecdysone, and that the active substances in the CNS were entirely different from those of the hemolymph and merely accelerated the release of the X-factors into the hemolymph. The other model suggested that the X-factors in the hemolymph and CNS were essentially identical substances that were produced in the brain, stored in the peripheral nerve terminals and released into the hemolymph under the influence of ecdysone. The experimental evidence obtained in this investigation support the latter scheme. Based on the results of the present work and the available information in the literature a comprehensive model for the control of puparium formation in fleshflies is presented in Figure 4.

According to this model, the retraction of the anterior segments, the longitudinal contraction to form a barrel shape, and the shrinkage of the cuticle that results in a smooth cuticle are under direct neural control of the CNS. The retraction of the anterior segments is also controlled indirectly through a neurosecretion from the CNS, as is also tanning. The neurosecretory material synthesized in the brain contains 2 distinct entities, the anterior retraction factor (ARF) which controls the retraction of the anterior segments and the puparium tanning factor (PTF) which controls tanning, and these neurosecretions are assumed to be stored in the peripheral nerve endings in all segments of the larva. The molting hormone, ecdysone, whose synthesis and liberation is controlled by another neurosecretion, ecdysiotropin, triggers the release of the neurosecretory materials from the peripheral nerves into the hemolymph. Ecdysone also induces the synthesis of various materials such as nucleic acids, enzyme proteins, tanning substrates and other cofactors which enable the released neurosecretory material to bring about retraction of the anterior segments and tanning. Thus, the CNS, both directly and indirectly controls the entire process of puparium formation in flies. Arguments in favor of this scheme will be presented in this discussion.

Of the various processes shown in the scheme, the prothoracotropic effects of neurosecretion and ecdysone-induced protein synthesis during pupariation are well documented in the literature. The involvement of both the CNS and what we now know as ecdysone during molting, and the relationships between the CNS and ecdysone were demonstrated in the pioneering work of Williams (1947). Reports on ecdysone-induced protein synthesis during pupariation, which is a special molting process, abound in the literature (Sekeris, 1965; Karlson and Sekeris, 1966; Sekeri, Sekeris and Karlson, 1968; Arking and Shaaya, 1969; Sahota and Mansing, 1970; Wyatt and Wyatt, 1971; Thomasson and Mitchell, 1972). However, except for the enzyme DOPA decraboxylase which is involved in tanning, the relationship of these proteins to the various morphological events that occur during pupariation is not clear. As will be explained later, at least some of these proteins might coordinate with ARF and PTF in bringing about the retraction of the anterior segments and tanning.

Though no experiments have so far been performed by us, it is clear from the studies of Osborne and his colleagues (Osborne, 1967; Finlayson and Osborne, 1968; Osborne, Finlayson and Rice, 1971) that neurosecretion is stored in nerve endings. Recently they have demonstrated (Osborne *et al.*, 1971) the existence of neurosecretory nerve endings in 3 insects, including the blowfly larva. They suggest that the site of release of neurosecretory material is covered only by a flimsy layer of connective tissue and that the material is probably released into the immediate vicinity of the muscle and diffuses into the hemolymph. They were not sure about the function of the neurosecretory material and assumed that it might have some trophic function.

What causes the release of neurosecretory material from the neurosecretory nerve endings and when it is released is little understood. Ecdysone has been shown to be capable of inducing the release of neurosecretory material from cultured brains of a cockroach (Marks, Itycheriah and Leloup, 1972). Besides, there have been reports on the action of ecdysone on the plasma membrane (Kambysellis and Williams, 1971b). In a previous report (Fraenkel *et al.*, 1972) it was demonstrated that the hormonal factors responsible for the acceleration of the retraction of the anterior segments and tanning can appear in the blood even in the absence of CNS. In that experiment larvae were ligated pre-critically (*i.e.*, no ecdysone was present in the system), and the blood in the ligated hind parts became active after ecdysone injection when the cuticle started to tan. Several experiments in the present investigation have indicated that the hormonal factors in the neurosecretory material of the CNS are essentially identical to those that appear in the hemolymph during pupariation (see below). This, coupled with the fact that the CNS from even much younger larvae (3 day old) contain accelerating factors (Zdarek and Fraenkel, 1969; Fraenkel *et al.*, 1972) tempts us to suggest that the

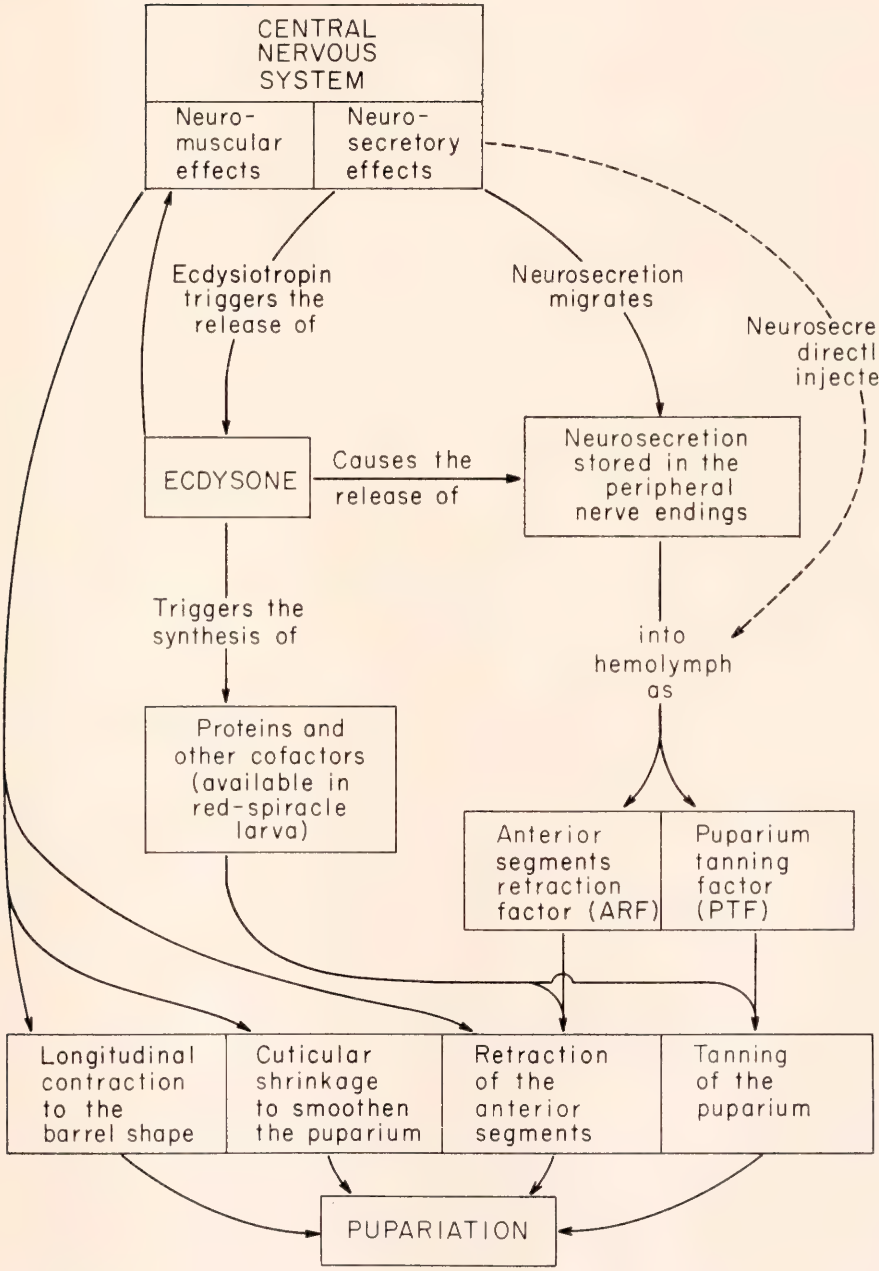
CENTRAL NERVOUS SYSTEM
Neuro-muscular effects
Neuro-secretory effects
Ecdysiotropin triggers the release of
Neurosecretion migrates
Neurosecretion directly injected
ECDYSONE
Causes the release of
Neurosecretion stored in the peripheral nerve endings
Triggers the synthesis of
into hemolymph as
Proteins and other cofactors (available in red-spiracle larva)
Anterior segments retraction factor (ARF)
Puparium tanning factor (PTF)
Longitudinal contraction to the barrel shape
Cuticular shrinkage to smoothen the puparium
Retraction of the anterior segments
Tanning of the puparium
PUPARIATION

FIGURE 4. A comprehensive scheme of the interrelationships between neuromuscular and neuroendocrine events that occur during puparium formation in the fleshfly *Sarcophaga bullata* P.; explanation in text.

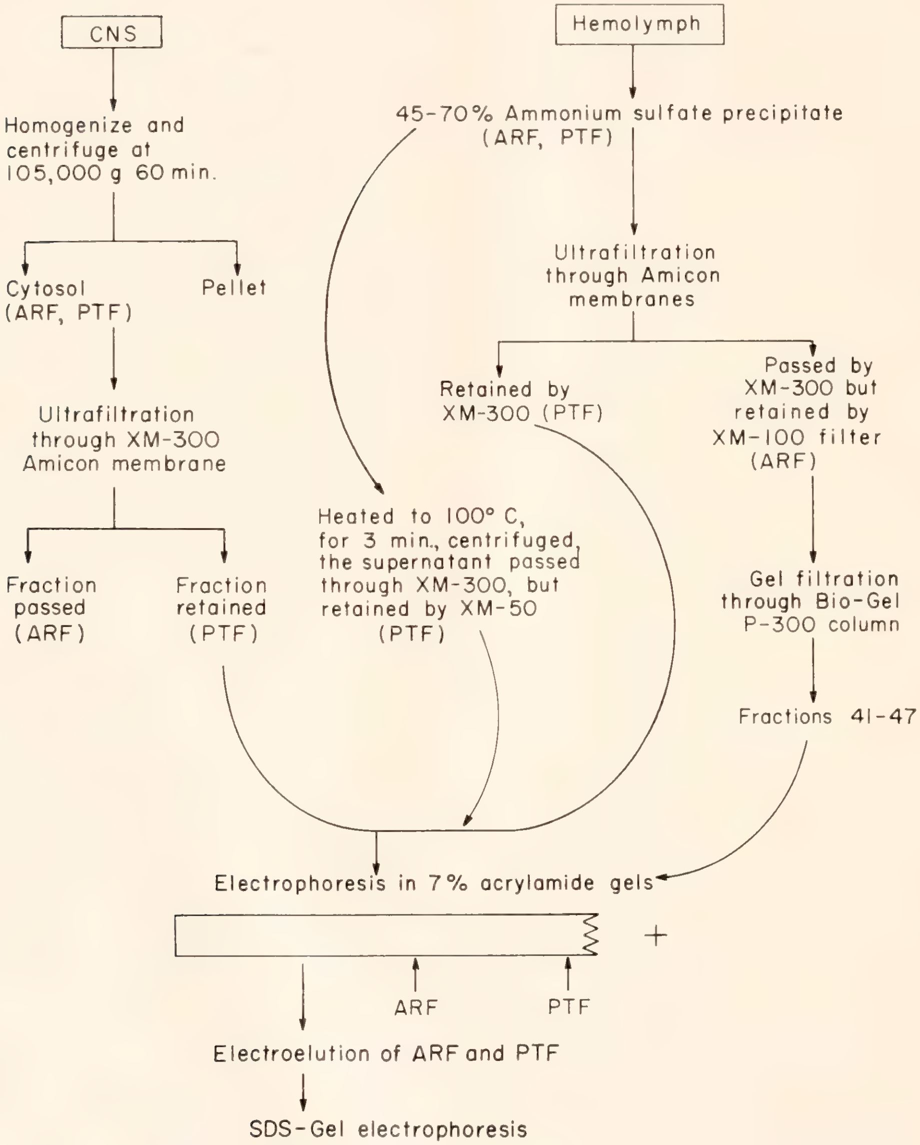
CNS
Homogenize and centrifuge at 105,000 g 60 min.
Cytosol (ARF, PTF)
Pellet
Ultrafiltration through XM-300 Amicon membrane
Fraction passed (ARF)
Fraction retained (PTF)
Hemolymph
45-70% Ammonium sulfate precipitate (ARF, PTF)
Ultrafiltration through Amicon membranes
Retained by XM-300 (PTF)
Passed by XM-300 but retained by XM-100 filter (ARF)
Heated to 100° C, for 3 min., centrifuged, the supernatant passed through XM-300, but retained by XM-50 (PTF)
Gel filtration through Bio-Gel P-300 column
Fractions 41-47
Electrophoresis in 7% acrylamide gels
+
ARF
PTF
Electroelution of ARF and PTF
SDS-Gel electrophoresis

FIGURE 5. Scheme of the steps taken in the isolation of ARF and PTF from the hemolymph and CNS of *Sarcophaga bullata*.

neurosecretory material containing these factors (ARF and PTF) is already stored in peripheral nerve endings in a pre-critical stage larva and is eventually released into the blood by the action of ecdysone prior to puparium formation. This assumption would be strengthened by a demonstration of the disappearance of neurosecretory material from the nerve terminals just prior to pupariation.

TABLE X

Comparison of the effects of various treatments on the retraction factor and tanning factor from the hemolymph of orange puparia of S. bullata

Treatment	ARF	PTF
(1) Heating at 100° C for 3 minutes	Activity lost	Activity remains
(2) Ultrafiltration	Activity passed through XM-300, but retained by XM-100 filter	Activity retained by XM-300 filter
(3) Acrylamide gel electrophoresis	Activity in the middle $\frac{1}{3}$ of the gel	Activity in lower $\frac{1}{3}$ of the gel
(4) Subunit molecular weight as determined by SDS-gel electrophoresis	90,000	26,000
(5) Injection of the purest fraction obtained by electroelution	Accelerates the retraction of anterior segments only	Accelerates tanning only

Direct and conclusive evidence has been obtained to show that there are two distinct factors for retraction and tanning. Figure 5 summarizes the various steps by which the separation of ARF and PTF from hemolymph, and PTF from the CNS was achieved. The properties of the two factors isolated from the hemolymph are different with regard to heating, ultrafiltration, electrophoresis, and subunit molecular weight (Table X).

The CNS extracts, when injected directly into the larva effect the retraction of anterior segments and tanning (Table II), the tanning effect being more predominant than the retraction effect. Nevertheless, the question arises as to whether the factors are identical to those in the hemolymph, and if so, why the tanning effect is predominant. Some of the characteristics which lend credence to the idea that the factors in the two tissues are identical have been listed in Table XI. How-

TABLE XI

Comparison of the properties of the ARF and PTF from hemolymph and CNS extracts

Property	Hemolymph		CNS	
	ARF	PTF	ARF	PTF
Ultrafiltration through XM-300 filter	Passed through filter	Retained by filter	Passed through filter	Retained by the filter
Heating at 100° C for 3 minutes	Destroyed	Not destroyed	Destroyed	Not destroyed
Acrylamide gel electrophoresis	Activity in middle $\frac{1}{3}$ of the gel	Activity in the lower $\frac{1}{3}$ of the gel	*	Activity in the lower $\frac{1}{3}$ of the gel

* Since the concentration of ARF was very low in the CNS extracts it was not isolated and hence the information is not available.

ever, there is a significant difference in heat resistance between CNS and hemolymph PTF's. That from the CNS is not broken down upon heating at 100° C whereas PTF from hemolymph is partially split (Table III). We do not know the reason for this difference.

Although the factors in the CNS and the hemolymph appear very similar, the relative quantities of ARF and PTF are quite different. In unfractionated hemolymph, injection of about 100 μg of protein accelerates retraction of the anterior segments in 50% of control time; but to induce any appreciable tanning activity at least 300 μg must be injected (Table I). However, it appears that the amount of CNS protein to affect PTF activity is one fifth of that which affects ARF activity. Hence, in the CNS the concentration of PTF must be higher than that of ARF and in the hemolymph the opposite must be true. We have no explanation as to why such differences in amount exist when they are secreted from one source.

The studies of Zdarek and Fraenkel (1969) demonstrated that the PTF of the CNS originates in the pars intercerebralis of the brain. Since their experiments were performed with ligated larvae, the existence of ARF in the CNS was not known at that time. However, as described above, ARF also is present in the cytosol fraction of the CNS and it is possible that it, too, originates from the pars intercerebralis. Ecdysiotropin and bursicon also originate from the same region. Are these two hormones related to either of our hormonal factors? It appears that with regard to ARF, the answer is an unequivocal no. ARF has a molecular weight of about 180,000 with two subunits of 90,000 each, whereas the molecular weights of ecdysiotropin and bursicon are well below 50,000. Furthermore, ARF is completely destroyed upon heating at 100° C for 3 minutes while the other two hormones of the brain are partially heat resistant.

What then, of the relationship between PTF and the other brain hormones? With a subunit molecular weight of about 26,000, and resistance to high temperature, it is possible that PTF may be related to ecdysiotropin. However, all previous attempts to isolate ecdysiotropin have ended up with multiple factors and the situation is presently too confusing to make any meaningful comparisons.

PTF as isolated from the CNS is most probably not related to bursicon. It is retained above the XM-300 filter even after heating and has a subunit molecular weight of about 80,000 which is about twice the size of bursicon.

However, the situation with regard to hemolymph PTF is slightly confusing. This fraction, which is initially retained by the XM-300 filter, is partially broken down when heated to 100° C and some of the activity then passes through the XM-300 filter and is retained by the XM-50 filter. After further purification by preparative acrylamide gel electrophoresis both heated and unheated PTF containing fractions appear to have a subunit molecular weight of 26,000. It appears that passage through the gel causes certain changes in the structure of the unheated high molecular weight material. One might argue that the hemolymph PTF has a molecular weight of about 52,000 with two subunits of 26,000 daltons, and that the subunits of this molecule are attached to a bigger molecule leading to its retention on the XM-300 filter when unheated. If this is true, then the question arises as to whether it might be related to bursicon which has a molecular weight of about 40,000 (Fraenkel, Hsiao and Seligman, 1966) and is also a tanning hormone secreted by the pars intercerebralis of the brain. The answer is most probably no because, (a) by heating at 100° C for 3–5 minutes 40–90% of bursicon activity is lost (Fraenkel *et al.,* 1966) but PTF activity is not (Table IV), and (b) in acrylamide gel electrophoresis bursicon appears at an R_f of 0.3–0.4; but PTF comes very close to the dye front.

Aside from the above, the injection of adult hemolymph active for bursicon into red-spiracle larvae did not accelerate tanning, and conversely, injection of PTF active hemolymph from orange puparia did not induce tanning in neck-ligated newly emerged adult flies (Zdarek and Fraenkel, 1969).

As shown in previous experiments, moderately pure samples taken from acrylamide gels are independent in their action. The ARF fraction effects only the retraction of anterior segments, and the PTF fraction effects only tanning. However, these factors are not effective in pre-critical stage larvae which lack ecdysone, in the same stage larvae when injected with subthreshold doses of ecdysone, nor when injected into early post-critical stage larvae (about 10–12 hours before pupariation). They are effective only if injected into red spiracle stage larvae.

Obviously, the active factors cannot replace ecdysone in effecting pupariation, and it is probable that they need ecdysone-dependent substances (*e.g.,* enzyme proteins, other co-factors), which become available only in the red-spiracle stage, but one can only speculate about the role of such substances in their action.

According to Kambysellis and Williams (1971a, 1971b, 1972), an undialyzable "macromolecular factor" from the hemolymph indispensable for the maturation of spermatozoa in the silkworm, *Samia cynthia,* is present in high titer in the hemolymph during and immediately after pupation. It is inactive in cultures of intact testes in the absence of ecdysone, but promotes spermatogenesis of germinal cysts if the cysts are removed from testes and then cultured. This suggested that the function of ecdysone here is to alter the permeability of the testis walls to facilitate the entry of the macromolecular factor. It is possible that during pupariation ecdysone might function in a similar way, aside from releasing the neurosecretory material from the neurosecretory nerve terminals.

To conclude our discussion of the scheme presented in Figure 4, there is now evidence of direct control by the brain of certain muscular events during pupariation, such as longitudinal contraction to the barrel-shape and cuticular shrinkage to smoothen the puparial surface (Sivasubramanian, Ducoff and Fraenkel, 1974).

Thus, the experimental evidence obtained in the course of this study confirms a previous report with regard to the existence of two different proteinaceous factors which separately effect retraction of the anterior segments and tanning during pupariation. It has also now been demonstrated that the factors in the hemolymph and CNS have a number of common properties, which makes it most probable that the source of the material in the blood is the CNS.

Even though the scheme in Figure 4 so far only applies to a single fly species, *Sarcophaga bullata,* we believe it to be valid for all cyclorrhaphous flies. It is also known that PTF occurs in the corpora cardiaca of *Periplaneta,* the brain of a bug, *Pyrrhocoris apterus,* and the brain of adult flies (Zdarek and Fraenkel, 1969), which suggests that it may play some role in insects which tan after molting. How far the model can be applied to other insects is difficult to predict at this point.

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SUMMARY

Injection of hemolymph from orange puparia or of CNS-extracts from larvae into red-spiracle larvae of *Sarcophaga bullata* accelerates the onset of pupariation in relation to retraction of the anterior segments and tanning. By the use of ammonium sulfate precipitation, heat treatment, ultracentrifugation, ultrafiltration, gel filtration, and electrophoresis, two proteinaceous factors were isolated and partially purified. One of these, called ARF (anterior retraction factor), accelerates retraction and has no tanning activity. The other, called PTF (puparium tanning factor), accelerates tanning and has no ARF activity. The ARF has a molecular weight of about 180,000, is heat labile, and was purified about 170 times. The PTF has a molecular weight of about 312,000, is heat stable, and was purified about 200 times. Similar factors were found in hemolymph and CNS-extracts, however, hemolymph was more active in ARF, and CNS in PTF. These factors are most probably not related to two other brain hormones, ecdysiotropin and bursicon, because of differences in molecular weights, electrophoretic mobilities, and distribution. Based on the similarities in the nature of the ARF and PTF in hemolymph and CNS it is suggested that they originate in the CNS, are stored in peripheral nerve endings, and released through the action of ecdysone into the hemolymph during pupariation.

The factors have no effect in the precritical (36 hours before pupariation) and early post-critical (10–12 hours before pupariation) stage larvae. They are functional only in a red-spiracle larva (2–4 hours before pupariation). This suggests that they cannot replace ecdysone in effecting pupariation, and that they require ecdysone-dependent enzyme proteins or other co-factors which become available only in the red-spiracle larva. The role of such substances may be to alter the permeability of target cell walls in order to facilitate the entry of ARF and PTF.

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INHIBITION OF NEMATOCYST DISCHARGE IN HYDRA FED TO REPLETION[1]

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The nematocysts of coelenterates are commonly assumed to be independent effectors; that is, each nematocyst discharges in direct response to environmental stimuli, usually a combination of mechanical and chemical factors provided by the prey (*e.g.* Pantin, 1942; Picken and Skaer, 1966). Though *stimulation* of discharge is apparently not influenced by the animal itself, there is some evidence that *inhibition* of nematocyst discharge is. The sea anemone, *Calliactis parasitica,* will use the nematocysts of the tentacles to make a preliminary adhesion to a whelk shell. Once a solid attachment is formed by the pedal disk, the tentacles will not react to a second whelk shell indicating an inhibition of discharge (Davenport, Ross and Sutton, 1961). Ross and Sutton (1964) demonstrated that the tentacles of the sea anemone, *Stomphia,* will not capture prey when swimming which, though not strictly proven, suggests a control of nematocyst discharge. More recently, Sandburg, Kanciruk and Mariscal (1971) have shown that the anemone, *Calliactis tricolor,* greatly reduces nematocyst discharge upon feeding to repletion. Similarly, hydra will no longer capture shrimp larvae after feeding to repletion (Hyman, 1940; Burnett, Lentz and Warren, 1960) although Burnett *et al.* (1960) did not attribute this phenomenon to the inhibition of nematocyst discharge. In this report, we have extended the description of nematocyst discharge behavior of hydra fed to repletion. The results indicate that inhibition of discharge does exist and is probably due to a rise in concentration of a metabolite(s).

MATERIALS AND METHODS

Hydra attenuata were used in all experiments. They were maintained in M solution (Lenoff and Brown, 1970), fed daily with *Artemia* nauplii, and washed 6–8 hours after feeding.

All feeding experiments were conducted with adult hydra having 1–2 buds. Each hydra was placed in 1 ml (in some experiments) or 10 ml (in others) of M solution. The hydra was offered a given number of shrimp, 1–10, depending on the experiment, which were placed repeatedly within the ring of tentacles until the animal caught them. Several minutes later (6–20), the number of dead and alive shrimp remaining outside the hydra was counted under a dissecting microscope (25 ×). From these numbers, the number killed and ingested was determined. After removal of the uningested shrimp, another group of shrimp (the same number as before) was offered. This procedure was repeated until the hydra would no longer ingest or kill shrimp, and the number captured was greatly reduced.

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Inhibition of the killing response, or stenotele discharge, was defined as five consecutive rejections of offered shrimp. A rejection was defined as a repeated contact (> 4) of one tentacle or three contacts of at least two tentacles by a shrimp which elicited neither a capture nor a killing response. Also, shrimp which were captured, escaped, and were still swimming normally 30 seconds thereafter were considered as rejected. At least two different shrimp had to be involved in the five consecutive rejections. Shrimp which contacted the tentacles and were not captured, but became immobile within 5–10 seconds were counted as killed.

For grafting experiments, hydra were bisected above the budding region, and the two apical halves were grated together by threading the pieces, back-to-back, on 12 lb nylon fishline (Stren, 0.013 in diameter). Sleeves of polyethylene tubing were placed at either end of the graft to prevent migration of individual pieces. After 60 minutes, the graft was removed to M solution and used in feeding experiments 4–7 hours later.

Nematocysts were discharged from the tentacles by electric shock as follows. Hydra were placed in M solution containing $2 \cdot 10^{-4}$ M fructose-1, 6-diphosphate (which enhances stenotele discharge; Lentz and Barnett, 1962) in a leucite chamber fitted with silver electrodes at two ends. Single pulses of current (40V, 5 mAmp, 13 msec duration) were delivered every 5 seconds by a Grass SD6 Student Stimulator. The percentage of stenoteles discharged was measured by counting the number of stenoteles in the tentacles before and after shocking as follows. Animals were placed in a drop of M solution on a glass slide, flattened slightly but not killed with a coverslip, and the visible stenoteles on the upper half of each tentacle counted with phase microscopy. The shocking procedure resulted in the discharge of 60% of the stenoteles in 10 minutes, and 90% in 40 minutes. By 40 minutes, about 50% of the other three nematocyst types were estimated to be discharged. The animals were not damaged by this procedure and were used for experiments 60 minutes after treatment.

Dilute and concentrated homogenates of *Artemia* were prepared as follows. Specimens of *Artemia* were centrifuged at 2000 *g* for 2 minutes. The loosely pelleted animals were transferred to a glass tissue homogenizer and homogenized. The homogenate was centrifuged at 300 *g* for 5 minutes to remove the skeletal parts. The resulting supernatant was termed dilute homogenate. The concentrated homogenate was prepared by pouring a thick suspension of *Artemia* onto a filter paper funnel to remove the fluid. The wet mass of shrimp was homogenized and the skeletal parts removed as before. Either homogenate was introduced into the gastral cavity of a hydra with a 10 μl Hamilton syringe tipped with a polyethylene needle. Normally, two injections spaced 15 minutes apart were made. After another 30 minutes, the ability of the animal to capture and kill shrimp was tested.

RESULTS

Pattern of inhibition of nematocyst discharge during feeding

To put the nematocyst discharge behavior during feeding in context, a description of the capture, killing and ingestion of prey by hydra is necessary. Prey (usually *Artemia* nauplii in the laboratory) swimming close to the tentacles contact the long cnidocils of the desmonemes and cause discharge of these nematocysts. Each nematocyst everts a short, thick thread which wraps itself around any rod-like object, such as, a spine on the swimming appendage of a shrimp. The trapped, struggling shrimp contacts the tentacle repeatedly and very soon hits the shorter cnidocil of a stenotele nematocyte. The nematocyst is discharged, everting a hollow thread which penetrates the shrimp, and a toxin is injected (Hessinger, Lenhoff and Kahan, 1973; Shapiro, 1968) which almost instantly paralyzes the shrimp, and probably kills it. Thereafter, the *Artemia* is transported to the hypostome by the tentacle and ingested. After taking in a number of shrimp by repeating this process several times, ingestion ceases. The number of shrimp ingested varies from day to day but for a group of animals of similar size is reasonably constant on a given day.

Since it is the stenotele and desmoneme nematocysts which are involved in feeding, the pattern of their discharge during the entire feeding process was first examined. Individual one-day starved hydra were placed in 1 ml M solution and a single *Artemia* placed within the crown of tentacles and the response noted. This was repeated until the animal no longer ingested or killed the shrimp larvae. A typical pattern with three or four stages emerged. At first, the starved animals quickly captured, killed, and ingested the shrimp. In contrast to earlier observations (Burnett *et al.*, 1960), shrimp were caught and killed on an out-stretched tentacle. Curling of the tentacle after capture to aid in bringing the shrimp in contact with a stenotele cnidocil did not seem to be necessary. After the ingestion of a number of shrimp, specimens of *Artemia*, were caught and killed but not ingested. Later shrimp were captured but not killed and eventually escaped. Such shrimp frequently struggled violently for up to two minutes making innumerable contacts with one or more tentacles before escaping. They were found to be swimming normally at least thirty minutes later indicating that they had not been struck by a stenotele nematocyst. This behavior indicates that discharge of the stenotele nematocyst had become inhibited. Finally, a stage was reached where shrimp could contact several tentacles in succession without capture. Though this was not carefully documented, and inhibition was not as complete as for stenotele discharge, a distinct inhibition of desmoneme discharge was apparent.

The behavior, or feeding activity, of six animals is shown in Figure 1 (solid line) in which each of the responses is given a numerical value: 3 = capture, kill and ingestion; 2 = capture, kill, but no ingestion; 1 = capture and escape; and 0 = no capture. The pronounced decreasing trend, which is typical, clearly depicts the change in response of the animal (= change in nematocyst discharge behavior) during the course of feeding. The increasing cessation of killing indicates that stenotele discharge becomes increasingly inhibited. The decreased activity and inhibition of nematocyst discharge is not due to a factor accumulating in the medium because the same behavior is obtained if the medium is changed after every five shrimp offered, or if the experiment is carried out in fifty ml of M solution. As a second measure of the increasing inhibition of stenotele discharge, the time required to kill a shrimp after capture was determined for each consecutive shrimp caught and killed by the animal. With increasing number of shrimp killed and ingested, the time of killing increases implying an increasing inhibition of stenotele discharge or a raising of the threshold for stenotele discharge.

Burnett *et al.* (1960) also found that after feeding to repletion killing of shrimp ceased and their capture occurred far less frequently than at the beginning of feeding

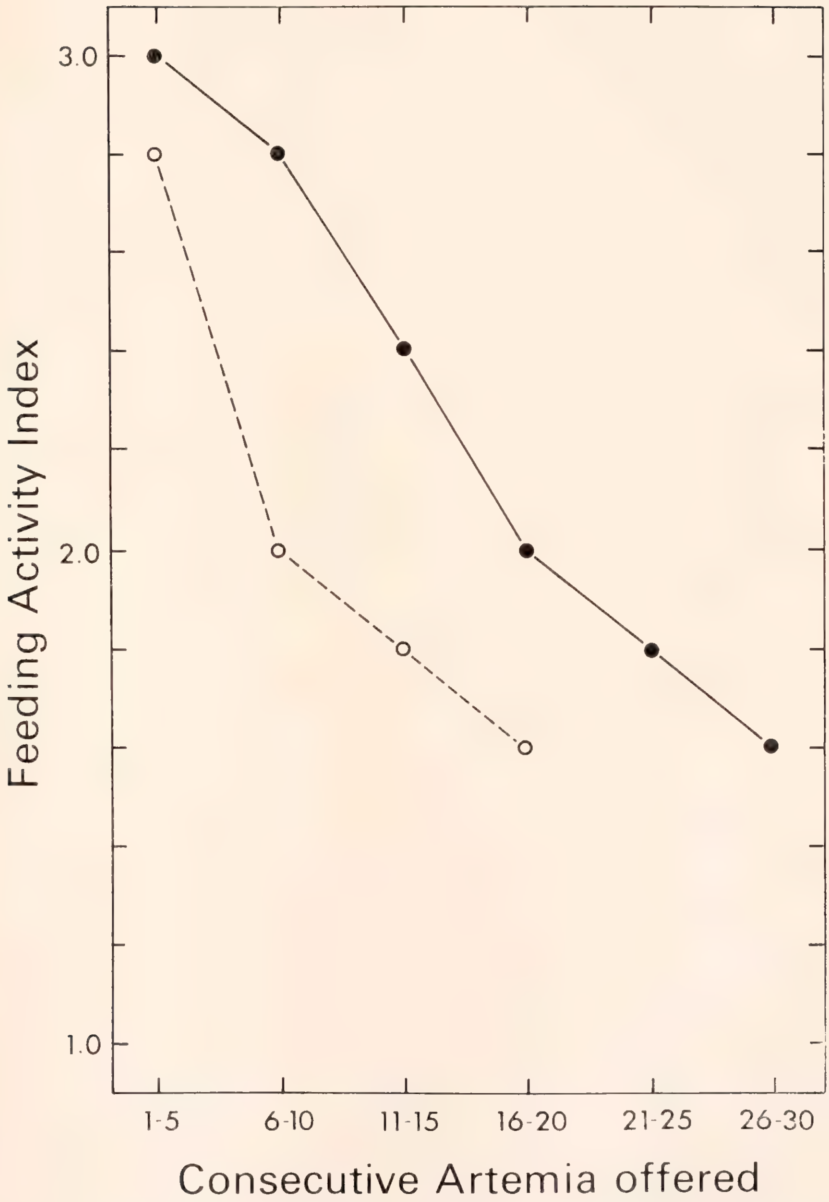

FIGURE 1. Quantitation of feeding behavior of hydra expressed as the feeding activity index. Individual hydra were offered one *Artemia* at a time. The response of the hydra to each *Artemia* was given a numerical value, the feeding activity index: 3.0 = capture, kill and ingestion of the shrimp; 2.0 = capture, kill, but no ingestion; 1.0 = capture and escape. Each point represents the average value of responses to five consecutive shrimp/hydra and the average of six hydra. Normal animals expressed by (filled circles); animals with a truncated gastral cavity by (open circles).

when the animal was starved. They have offered the interesting interpretation that the lack of capture was not due to the inhibition of desmoneme discharge, but instead, was due to the rapid release of the discharged desmoneme from the tentacle. Further, the cessation of killing was not due to the inhibition of stenotele discharge but rather due to the lack of opportunity of fully active stenoteles to fire since the shrimp were rapidly lost from the tentacles. Normally nematocysts are discarded after discharge, but only after enough time has elapsed (2–5 minutes) so that the captured and killed prey can be ingested. If a rapid release existed, then the time a captured shrimp remained attached to the tentacle after repletion should be much shorter than before repletion. This possibility was examined in *H. attenuata* by measuring the time elapsed between capture and kill before and after repletion, and the time between capture and escape after cessation of killing had set in. As shown in Table I, the time that a captured shrimp remained actively struggling on the tentacle after repletion and cessation of killing was longer than before repletion. Instead of a rapid release, captured shrimp remain attached to the tentacle longer after feeding to repletion in *H. attenuata*. Thus, cessation of killing is not due to lack of contact of the shrimp with active stenoteles, but due to the inhibition of stenotele discharge. Similarly, lack of capture sometime after feeding to repletion is probably due to cessation of desmoneme discharge instead of a rapid release of discharged desmonemes.

TABLE I

Length of time captured Artemia *remain attached to tentacles at three stage of feeding activity. Individual hydra were offered four shrimp at a time and the time elapsed between capture and kill or escape measured with a stop watch. Each time value is the average elapsed time for the indicated number of shrimp captured. The error is expressed as the mean deviation*

Expt.	Hydra	Capture, kill & ingestion		Capture, kill & release		Capture & escape	
		Number of shrimp	Time (sec)	Number of shrimp	Time (min)	Number of shrimp	Time (min)
1	1	15	20.7	—	—	3	38.3
	2	14	47.9	1	130	5	47.4
	3	13	54.5	2	36.5	5	44.8
	4	10	42.6	7	74.7	7	185.3
	5	12	128.6	1	42	7	38.3
	Average		58.9 ± 27.9		70.8 ± 31.5		70.8 ± 45.8
2	1	20	16.3	16	31.6	4	60.8
	2	11	9.6	23	19.4	5	11.0
	3	20	11.8	20	40.5	4	29.0
	4	12	8.0	30	24.6	—	—
	Average		11.4 ± 2.6		29.0 ± 7.0		33.6 ± 18.1

Three other aspects of the nematocyst discharge pattern were examined. Repletion in the animal could be a response simply to the number of shrimp ingested, or it could involve a time factor. Thus, cessation of ingestion may set

TABLE II

Effect of the amount of time elapsed on the total number of Artemia *ingested. Error is the mean deviation*

Feeding regime	Expt.	Number of hydra	Average number of shrimp ingested
One shrimp at a time	1	8	6.4 ± 2.6
Two shrimp, a 1 hour pause, then one shrimp at a time		7	9.3 ± 2.9
Four shrimp, a 1 hour pause, then one shrimp at a time		7	9.1 ± 1.0
Fed to repletion, a 2 hour pause, fed again until repletion	2	12	18.4 ± 5.4
Fed 6 shrimp, a 2 hour pause, fed until repletion		14	16.6 ± 2.7

in after feeding for a certain time period, regardless of the number of shrimp ingested. To decide between these alternatives, animals were fed under different regimes in two experiments as shown in Table II. In each experiment, the hydra reached repletion after having ingested a similar number of shrimp even though the total period of time until repletion was reached was different. Thus, the time interval from the onset of feeding to repletion does not play a role. The difference between the two experiments in the total number of shrimp ingested per hydra reflects the day-to-day variability in the number of shrimp a hydra will consume.

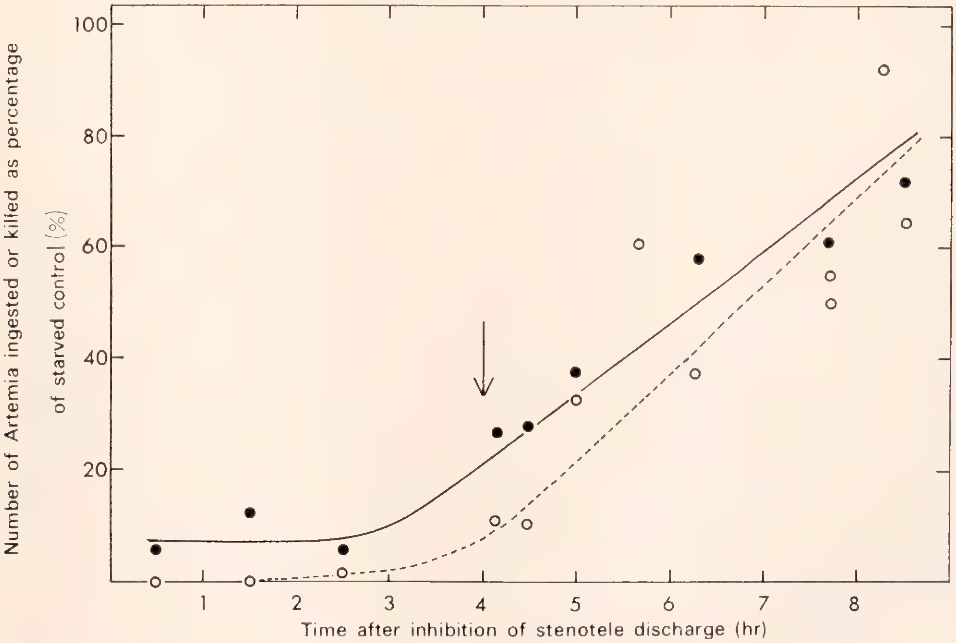

FIGURE 2. Kinetics of recovery from inhibition of stenotele discharge. The results of two experiments are presented together. Each point is the average of 4–6 hydra. The two curves are the percentage of *Artemia* killed (filled circles) and the percentage ingested (open circles) compared to starved controls.

How long stenotele discharge remains inhibited, once inhibition was reached, was also investigated. Animals were fed to inhibition. Then, at intervals over a period of several hours, a group (different group for each interval) of animals was tested for their ability to ingest and kill shrimp. They were offered shrimp until cessation of killing set in. As shown in Figure 2, the animals do not ingest any and kill very few shrimp until they regurgitate the remainder of their previous meal, which occurs about four hours after feeding to repletion. Thereafter, the recovery to the state of a starved animal requires an additional four to five hours. A pronounced change from severe inhibition to a state of some killing and ingestion takes place shortly after regurgitation.

Finally, the question arose as to whether or not the stenoteles mounted along the body column, which are not involved in the feeding process, were also inhibited from discharging after feeding to repletion. These stenoteles will also kill shrimp upon contact but do so without previous capture since there are no desmonemes mounted on the body column. The behavior of these stenoteles was examined by offering shrimp to the bodies of starved and repleted animals and then measuring the time required for the body to kill 15 shrimp. In the process of offering the shrimp to the body, many were caught and killed by the tentacles, which proved to be a useful internal control. As seen in Table III, there is a striking difference in the behavior of body and tentacle stenoteles in repleted animals. The bodies of one-day starved hydra killed the 15 shrimp 3–4 times faster than did the bodies of repleted animals. Thus, the body stenoteles of the repleted animals were inhibited to an extent. However, they were far less inhibited than the stenoteles of the tentacles. During the same time period, the stenoteles of the starved animals killed at least 10 times as many shrimp as the tentacles of repleted animals, and probably more since there was no room for more than forty shrimp on the tentacles. Thus, the inhibition of discharge affects those stenoteles involved in feeding more strongly.

TABLE III

Effect of feeding to repletion on the discharge of stenoteles mounted on the body column. Each hydra was offered about 120 specimens of Artemia *and the time required for the body to kill 15 shrimp measured. The number of contacts of shrimp with the body and tentacles were measured on average as 20 contacts/min for the tentacles and 12 contacts/min for the body. The error is expressed as the mean deviation*

State of feeding	Number of hydra	Average time required for body to kill 15 *Artemia* (min)	Number of shrimp killed by tentacles in the time required for body to kill 15 shrimp
Starved	5	5.4 ± 2.5	~40
Fed to repletion	14	19.5 ± 4.1	4.9 ± 2.0

The inhibition of nematocyst discharge is due to a gastral cavity filled with shrimp

The foregoing experiments indicate that the ability to discharge stenoteles and desmonemes is sharply inhibited in animals fed to repletion. The inability to kill more shrimp could be due merely to an exhaustion of fully mature stenoteles or an inhibitory feedback effect caused by discharging a given number of the stenoteles in the tentacles. A lack of mature stenoteles seems unlikely in the light of an experiment by Burnett *et al.* (1960) which we corroborated. If the captured and killed shrimp are removed from the tentacles, thus preventing ingestion, a hydra will kill at least twice as many than if allowed to ingest them. Thus, the tentacles contain at least twice as many mature stenoteles as are used at the time of inhibition of killing. Also, there is no apparent feedback on discharge due to a loss of the stenoteles.

The availability and feedback arguments were examined more directly in the following experiments. First, the percentage of stenoteles used to feed to inhibition was determined by counting the number of stenoteles in the tentacles before and after feeding as described in Materials and Methods. As shown in Table IV, 21% of the stenoteles had been used. Sixteen per cent were used in a second experiment. If these percentages represented either the number of available mature stenoteles or the percentage of stenoteles discharged required to achieve inhibition of discharge by a feedback mechanism, then removal of 50% of the stenoteles before feeding should completely prevent killing of prey. This possibility was tested as follows. Two groups of hydra were selected. The stenoteles of one group were counted with the coverslip method. Then, both groups were subjected to electric shock, as described in Materials and Methods, for various periods of time (5–15 minutes). The group of animals previously counted were counted again. More than 60% of the stenoteles had been discharged. The second group of animals was placed in M solution, allowed to recover from the treatment for two hours, and then offered shrimp. As shown in Table V, they killed and ingested a normal number of shrimp. These results argue against availability of mature stenoteles and feedback due to a number of discharged stenoteles as the cause of the observed

TABLE IV

Percentage of stenoteles discharged by animals fed to inhibition. All errors are expressed as the mean deviation

Hydra	Number of *Artemia*		Average number of stenoteles/½ tentacle*		Percentage of stenoteles discharged (%)
	Killed	Ingested	Before feeding	After inhibition	
1	30	12	93.6 ± 30.9	64.2 ± 7.4	31.4
2	27	16	53.6 ± 9.1	52.0 ± 9.4	3.0
3	27	15	66.2 ± 10.9	50.8 ± 12.5	23.2
4	23	10	63.2 ± 12.7	34.6 ± 6.4	45.2
5	20	11	56.8 ± 6.6	39.8 ± 7.3	30.0
6	28	23	66.6 ± 12.7	47.4 ± 4.7	28.8
7	21	17	53.0 ± 11.2	45.2 ± 15.0	14.7
8	23	16	51.2 ± 7.6	35.7 ± 6.8	30.3
9	26	18	55.0 ± 10.2	51.0 ± 13.2	7.3
10	15	10	34.0 ± 7.3	33.6 ± 11.7	0.1
				Average	21.1 ± 12.1

* Each value is the average of 4–6 ½-tentacles.

TABLE V

Effect of large numbers of discharged stenoteles on feeding behavior. Errors are expressed as the mean deviation

Expt.	Length of electric shock treatment (min)	Animals assayed for feeding behavior			Animals assayed for number of stenoteles after electric shock	
		No. Hydra	Average number of shrimp		Number of hydra	Percentage of stenoteles remaining (%)
			Killed	Ingested		
1	0	4	29.5 ± 1.5	19.0 ± 0.5	—	(100)*
	10	4	27.5 ± 2.2	17.5 ± 2.2	3	36.4
	15	4	25.0 ± 3.0	20.0 ± 3.0	3	21.9
2	0	4	14.8 ± 1.7	12.0 ± 1.5	—	(100)*
	5	4	17.2 ± 4.8	14.5 ± 3.2	1	87.8
	10	4	10.8 ± 3.2	9.2 ± 4.8	1	42.0
	15	4	11.0 ± 2.5	8.0 ± 1.5	1	43.2

* 100% was assumed, not measured. Percentage of stenoteles remaining

$$= \left(\sum_{i=1}^{N} \frac{\text{Stenoteles}/\frac{1}{2}\text{ Tentacle After}}{\text{Stenoteles}/\frac{1}{2}\text{ Tentacle Before}} \Big/ N \right) \times 100$$

cessation of killing and support the view that stenotele discharge has become inhibited.

To obtain evidence that the inhibition of discharge was directly related to the gastral cavity filled with shrimp, the following pair of experiments were carried out. If a filled gastral cavity is related to inhibition, then an animal with a smaller gastral cavity should ingest fewer shrimp and display the observed pattern of nematocyst discharge inhibition (Fig. 1) after a smaller number of offered shrimp than a normal animal. To test this possibility, animals were bisected beneath the ring of tentacles, the apical part allowed to heal for 24 hours, and then offered *Artemia* one at a time. As expected these smaller animals ingested fewer shrimp compared to normal animals (Table VI), and they displayed the cessation of killing and capturing behavior much earlier than the normal animals [compare smaller animals (dotted line) with normal animals (solid line) in Fig. 1]. These results indicate that the filled volume, but not the absolute number of shrimp, is significant.

In a second experiment, two one-day starved animals were bisected in the mid-gastric region and grafted together as shown in Figure 3. The graft was allowed

TABLE VI

Number of shrimp ingested as a function of the size of the gastral cavity; Group A = animals bisected beneath ring of tentacles and allowed to heal; Group C = control. Errors expressed as the mean deviation

Expt.	# Hydra	Group A	Group C
1	12	2.9 ± 0.6	26.9 ± 2.4
2	12	4.5 ± 0.7	13.0 ± 1.5
3	8	5.3 ± 0.8	24.0 ± 1.1

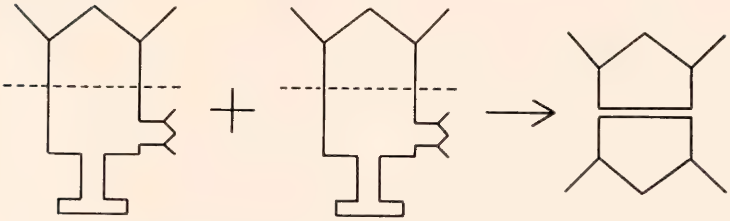

FIGURE 3. Grafting procedure for double-headed animals.

to heal (which requires about 1-$\frac{1}{2}$ hours; Bibb and Campbell, 1973) and then fed 4–7 hours later. In these experiments, the two heads had a common gastral cavity; that is, a wall had not yet formed between the two halves. If both heads of a starved animal were offered shrimp simultaneously, both killed and ingested shrimp in equal amounts indicating that the graft combination had no effect on nematocyst discharge in either head. If one head was fed to repletion and inhibition of killing, and then, the second head fed, the second head ingested essentially no shrimp and captured or killed $\frac{1}{4}$–$\frac{1}{5}$ as many as the first head (Table VII). A similar effect was found, but not as pronounced if a wall partitioning the cavity existed. This indicates the inhibition need not be transmitted through the gastral cavity alone. Clearly, the presence of the shrimp in the gut caused the inhibition of the discharge of stenotele nematocysts in the second head.

These results taken together with those of the earlier experiments provide more evidence that inhibition of stenotele discharge is the reason the killing of shrimp ceases after feeding to repletion and that a filled gastral cavity is in some way responsible for the inhibition of stenotele discharge. This result is not unexpected since animals normally cease eating when their stomachs are full.

TABLE VII

Effect of filled gastral cavity on the feeding behavior of the second head of a two-headed animal. Errors expressed as the mean deviation

Animal	Time after graft (hrs)	First head: number of shrimp			Second head: number of shrimp		
		Captured	Killed	Ingested	Captured	Killed	Ingested
1	4	15	13	11	10	7	0
2	4	9	7	3	9	3	0
3	4	16	14	9	4	2	0
4	6	21	17	11	2	2	1
5	6	24	20	13	5	4	0
6	6	18	18	8	3	1	0
7	6	19	19	14	2	1	0
8	6	26	23	12	6	2	1
9	6	20	20	7	6	4	0
10	6	26	24	10	3	1	0
11	7	21	17	10	3	2	0
12	7	11	11	5	3	3	1
13	7	15	9	3	3	3	0
Average		18.5 ± 4.2	16.3 ± 4.2	8.9 ± 3.0	4.5 ± 2.0	2.7 ± 1.2	0.23 ± 0.35

Properties of a filled gastral cavity responsible for inhibition of nematocyst discharge

The next question that arose was what kind of information is transmitted from filled gastral cavity to the nematocysts in the tentacles. In animals higher on the evolutionary scale, several different kinds of mechanisms involved in satiation or the cessation of ingestion are known to exist. One is based on the physical distention of the walls of the stomach (Janowitz and Grossman, 1949), which in several species of insects, is monitored by stretch receptors (Finlayson and Lowenstein, 1958). A second is based on the levels of metabolites, such as glucose, in the blood (Mayer and Thomas, 1967). As another, Mook (1963) has suggested the involvement of osmoreceptors in the stomach. Also, controls of ingestion related to the physical process of eating have been shown to exist (Grossman, 1955; LeMagnen, 1971). In analogy with other animals cessation of ingestion and inhibition of nematocyst discharge in hydra may be based on one or more of these mechanisms.

TABLE VIII

Effect of extreme distention of the body wall on feeding behavior. Feeding was initiated 0–25 minutes after injection. The variable starting time had no effect. The errors are expressed as the mean deviation

State of body wall	Number of hydra	Number of shrimp		
		Captured	Killed	Ingested
Control	3	12.0 ± 2.7	11.3 ± 3.1	9.0 ± 0.7
Distended	7	14.3 ± 4.1	11.1 ± 3.0	8.6 ± 1.8

To test the idea that the hydra recognizes physical distension of the body wall, an air bubble (1.0–1.5 μl) was injected into the gastral cavity of a hydra with a microliter syringe tipped with a polyethylene needle. The bubble, with a volume 10 times greater than the empty gastric cavity (0.1 μl, Bode, unpublished results), extended the walls more than those of an animal fed to repletion. The hydra was then offered 3–4 *Artemia* at a time and the response noted. The results Table VIII) indicate that the hydra behaved as a starved animal killing and ingesting as many shrimp as a one-day starved animal. With the increasing number of shrimp ingested, the bubble decreased in volume, but the total extended volume of the gastral cavity remained unchanged. These results indicate that severe distention of the body walls surrounding the gastral cavity has no effect on killing, hence on stenotele discharge in the tentacles. Thus, it is unlikely that the nature of the formation is due to mechanical stretching. Burnett *et al.* (1960) carried out a similar experiment by injecting glass beads into the gastral cavity and reported that the animals killed half as many shrimp as did a set of control animals. The discrepancy between the two results may be more apparent than real for the experimental animals of Burnett *et al.* (1960) were compared with control animals tested in a previous experiment, and as noted above, the number of shrimp killed and ingested can vary greatly from day to day.

An observation made in an experiment described earlier supports the conclusion that the distended body wall of the gastral cavity is not responsible for the inhibition of stenotele discharge. Animals fed to repletion have a fully extended gastral cavity. Four hours later, shortly before regurgitation of the remaining food, the volume of the gastric cavity had returned close to that of a one-day starved animal, yet the animal killed very few of the offered shrimp; that is, stenotele discharge was still inhibited to a large degree. If simple physical distention of the body wall were related to inhibition of stenotele discharge, we would have expected the extent of stenotele discharge, we would have expected the extent of stenotele discharge to be more like that of a starved animal than like a repleted one.

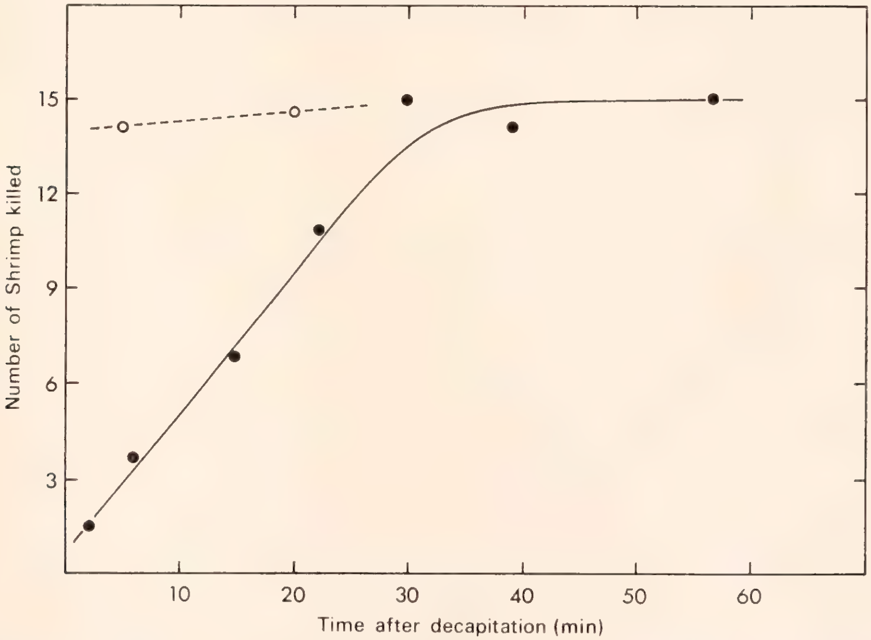

FIGURE 4. Decay of inhibition of stenotele discharge in severed heads. At each time interval after severance, a group (3–5) of hydra were offered 15 shrimp and the number killed within two minutes noted. The two curves are for heads of repleted animals (filled circles) and for heads of starved animals (open circles).

A related possibility concerns "neck formation" in the animal. Some time after feeding to repletion, the portion of the body column directly beneath the ring of tentacles elongates, that is, stretches, becoming very narrow like a neck (Blanquet and Lenhoff (1968). A stretch receptor located in this region could monitor ingestion and hence be involved in the control of nematocyst discharge. However, this is unlikely because neck formation takes place after inhibition of stenotele discharge sets in.

Since physical distention of the body walls is probably not involved, the inhibition of stenotele discharge may be due to the accumulation of a specific metabolite somewhere in the animal or to a general osmotic effect due to the accumulation of ions and metabolites in the gastral cavity. If so, gradual changes in inhibition might be expected as the level of (say) the metabolite rose or fell in the animal. The slow recovery from inhibition by animals after regurgitation of the remainder of the previous meal is consistent with this view, as are the three following experiments.

Removing the head from the filled distended body of a repleted hydra releases the inhibition of stenotele discharge to some extent after 15 minutes (Burnett *et al.*, 1960). This was examined more fully by decapitating hydra, and thereafter, perioically offering 15 shrimp to each of a group of severed heads (a different group was used for each time point) and noting how many were killed within two minutes. The head of a starved animal killed *Artemia* immediately after severance and behaved as an intact animal by killing essentially all shrimp offered (Fig. 4). Thus, simply the removal of the head, as was expected from earlier work (*e.g.*, Pantin, 1942), had no effect on stenotele discharge. In contrast, a head removed from a repleted animal killed only one or two shrimp in two minutes. Hence, stenotele discharge was still inhibited. With increasing time after severance, the head killed more of the offered shrimp, and by 30 minutes, behaved as a severed head of a control animal. The result indicates that the inhibition of discharge decays slowly which is consistent with the gradual loss of a metabolite by either degradation or leakage.

TABLE IX

Effect of different feeding regimes on the lag time between repletion and cessation of killing. Errors are expressed as the mean deviation

Number of hydra	Number of shrimp offered at a time before repletion	Time required to feed to repletion	Number of shrimp offered at a time after repletion	Lag time between repletion and inhibition	Number of shrimp killed after repletion
5	10	25.0 ± 1.6	10	20.0 ± 3.2	9.2 ± 1.4
5	10	27.2 ± 5.0	2	19.0 ± 6.8	5.8 ± 2.2
5	2	67.4 ± 5.1	10	13.6 ± 3.7	13.2 ± 6.4
4	2	93.5 ± 8.2	2	6.5 ± 3.8	2.8 ± 1.6

The lag time between cessation of ingestion and inhibition is also of this general length suggesting that the inhibition of stenotele discharge sets in gradually. If the lag time is due to a gradual rise in the concentration of a metabolite, then the feeding regime (the number of shrimp offered and ingested per time interval) may affect the length of the lag time. To examine this, hydra were fed either two or ten shrimp at a time until repletion. As was expected (Table IX), the time required to reach repletion was three times longer for animals fed two shrimp at a time than for those fed ten at a time since repletion is a function of a gastral cavity filled with shrimp. Thereafter, animals fed to repletion of both groups were offered shrimp at short intervals until killing ceased. The lag time was, indeed, affected by the initial feeding regime. Animals fed to repletion by offering shrimp ten at a time had a lag of ~ 20 minutes compared to ~ 10 minutes for those offered two at a time (Table IX). The difference in lag times is consistent with the view that a certain amount of metabolism must take place before the concentration of some metabolite reaches levels which inhibit stenotele discharge.

Hydra fed ten shrimp at a time reach repletion in 25 minutes, but the digestion must continue another twenty minutes before the concentration is high enough. Animals fed shrimp two at a time require longer to reach repletion (~ 75 minutes) and, therefore, have more time to accumulate a metabolite(s). This would explain why the lag time between repletion and inhibition of killing is shorter in this case.

Further, if the lag time is a function of metabolism, the length of the lag time should be independent of the number of shrimp killed after repletion has set in. This point was examined in the same experiment. As shown in Table IX, animals fed to repletion by either regime were then offered either two or ten shrimp at short intervals until killing ceased. Those hydra offered 10 shrimp at a time killed twice as many as those offered two at a time indicating that the numbers of shrimp killed after repletion have no effect on the lag time.

TABLE X

Effect of two concentrations of homogenates of Artemia *injected into the gastral cavity on the feeding behavior. Errors are expressed as the mean deviation*

Expt.	Injected solution	Number of hydra	Number of shrimp		Feeding activity of experimental animals as compared to controls (%)	
			Killed	Ingested	Killed	Ingested
1	M solution	5	20.8 ± 3.8	8.2 ± 2.6		
	Dilute homogenate	5	14.8 ± 4.7	2.0 ± 1.6	71	24
2	M solution	4	17.5 ± 3.0	7.5 ± 1.8		
	Dilute homogenate	4	9.5 ± 4.0	0	54	0
3	M solution	5	20.2 ± 1.4	8.8 ± 0.7		
	Concentrated homogenate	7	5.1 ± 1.9	0	25	0
4	No injection	4	22.5 ± 2.0	10.2 ± 1.8		
	M solution	6	20.8 ± 2.6	9.5 ± 2.5		
	Concentrated homogenate	7	7.4 ± 2.5	0	35	0

Finally, if inhibition of stenotele discharge is due to a metabolite either obtained directly from the *Artemia* or by metabolizing a component of the *Artemia*, then the degree of inhibition may be a function of the concentration of the original metabolite in the gastral cavity. This was tested as follows. Two different concentrations of *Artemia* homogenate were prepared as described in Materials and Methods. Two injections, 15 minutes apart, of either homogenate into the gastral cavity yielded hydra that had the extended appearance of animals fed to repletion. Thirty minutes later, these animals were offered shrimp four at a time and their ability to kill and ingest the shrimp analyzed in the usual manner. As shown in Table X, the dilute homogenate inhibited killing by only 30–50% as compared to controls injected with M solution, whereas the concentrated homogenate inhibited ingestion completely and inhibited killing by 65–75%. In experiment 4, some animals that had not been injected were also tested, and it is evident that injection as such has no effect on the ingestion and killing behavior. These results are consistent with the hypothesis that a metabolite(s) is involved and supports an experiment carried out by Burnett *et al.* (1960). They injected homogenates of starved hydra and hydra repleted with shrimp and found that the latter, but not the former, reduced the killing of shrimp. The fact that the dilute homogenate, estimated to be 5–10 times less concentrated than the concentrated homogenate, is significantly less effective suggests that the metabolite(s) must be present in rather high concentration. Also, these results underscore the earlier result that the distended body walls are not responsible for the inhibition of nematocyst discharge since the dilute and concentrated homogenates extended the body walls to the same degree but have different effects on the killing activity.

Discussion

The results presented above indicate that stenotele, and to a lesser extent desmoneme, discharge can be inhibited by a hydra fed to repletion. This adds to the increasing body of evidence (see Picken and Skaer, 1966) that nematocytes are not completely independent effectors. It has been suggested (Burnett *et al.,* 1960) that the inhibition in hydra is more apparent than real because after an animal has fed to repletion, desmonemes discharged while contacting shrimp are released so rapidly that contact with the still active stenoteles is avoided. For *H. attenuata,* the length of time between discharge and release of desmonemes does not change after reaching repletion, yet the killing of *Artemia* ceases, and, in time, far fewer are captured indicating that the discharge of stenoteles and desmonemes has become inhibited.

This effect is not due to a lack of available mature stenoteles and desmonemes nor due to feedback inhibition based on the number of nematocysts discharged for the following reasons: (a) the animal will kill more shrimp than normal if prevented from ingesting captured and killed shrimp; (b) removal of more than half the available stenoteles has no effect on feeding whereas feeding to repletion requires less than 20% of the stenoteles; (c) animals with a truncated gastral cavity but normal set of tentacles will capture and kill far fewer shrimp than an animal with a normal-sized gastral cavity; (d) if the first head of a two-headed animal with a common gastral cavity is fed to repletion and inhibition of killing, the second head will kill very few shrimp even though it has a normal complement of stenoteles in the tentacles. All of these experiments strongly support the original indications that stenotele discharge is inhibited after feeding to repletion.

Other observations suggest that there are a variety of circumstances in which a hydra will not capture and kill shrimp, and indicating an inhibition of nematocyst discharge. Hydra subjected to electrical shock will not capture shrimp for 30 minutes after treatment. Animals squashed mildly under a coverslip will not feed for 60 minutes thereafter. Similarly, in a stock culture, some animals will feed normally and others will not feed at all. These observations are understandable in the teleological sense that a "sick" hydra or one fed to repletion has no need for food and, hence, will inhibit the discharge of nematocysts involved in obtaining food. In this connection, it is interesting that stenoteles mounted on the body column which are not involved in feeding are inhibited far less (~ 5 times less) than those mounted in the tentacles. This suggests that the mechanism of inhibition is somewhat specific for tentacle stenoteles. It is not known if the discharge of the two types of isorhizas are subject to inhibition.

As to possible mechanisms underlying the inhibition of stenotele and desmoneme discharge, some information has been obtained. The experiments with two-headed animals and those with truncated gastral cavity [(c) and (d) in an earlier paragraph of the discussion] demonstrate that inhibition is correlated with a gastral cavity filled with shrimp, but is independent of the absolute number of shrimp ingested. Though an animal with a filled gastral cavity has greatly distended body ways, suggesting physical distention as the mechanism, this is not the case. Animals fed to repletion 3 to 4 hours previously no longer have a distended gastral cavity, yet stenotele discharge was still severely inhibited. Also, distending the body walls with an air bubble does not cause inhibition and, in fact, has no effect on feeding at all. Finally, two different concentrations of *Artemia* homogenate injected into the gastral cavity, though distending the body walls to the same extent, had markedly different effects on the number of shrimp killed and ingested during subsequent feeding.

The results of other experiments are consistent with a metabolite(s) as the basis of the mechanism of inhibition. The lag time between cessation of ingestion and inhibition of stenotele discharge is of the order of 10–20 minutes while the decay time of inhibition in severed heads is about 30 minutes. These gradual processes are much too long to be based on mechanisms involving the nervous system but are consistent with slow rise or fall in concentration of a metabolite. Further, the fact that the concentrated *Artemia* homogenate was far more effective than the dilute homogenate in inhibiting stenotele discharge indicates not only that the *Artemia* provided some component that caused inhibition but that the component must be in relatively high concentration. These data suggest that the *Artemia* provides a (or several) specific metabolies(s) that is directly involved or is altered by the hydra and then involved in the inhibition of nematocyst discharge. The results are also consistent with an inhibition based on an osmotic effect due to a general rise in all metabolite and ion concentrations.

The logical extention of this work is to determine whether inhibition is due to a general osmotic effect or due to a threshold concentration of a specific metabolite(s). The answer to this question will help clarify whether or not the basic mechanisms involved in satiation in hydra and more highly evolved animals are similar.

SUMMARY

1) The pattern of nematocyst discharge was observed in hydra fed shrimp until repletion. Some time after cessation of ingestion stenotele discharge ceases, and at a later time desmoneme discharge is greatly diminished. Lack of capture of shrimp is not due to a rapid release of desmonemes in animals fed to repletion.

2) Stenotele discharge remains severely inhibited until regurgitation of the remainder of the previous meal. Thereafter, stenotele discharge becomes progressively less inhibited and is normal 4–5 hours later.

3) Discharge of stenotele mounted on the body column is partially inhibited in animals fed to repletion, but far less inhibited than for the stenoteles in the tentacles.

4) Cessation of stenotele discharge after feeding to repletion is not due to the absence of mature stenoteles, as there are at least twice as many mature stenoteles present in the tentacles as are needed to feed to repletion.

5) Inhibition of stenotele discharge is correlated with a gastral cavity filled with shrimp, but is not correlated with the absolute number of shrimp ingested.

6) Physical distention of the gastral cavity due to the ingested shrimp is not the cause of inhbition of stenotele discharge.

7) The changes in inhibition of stenotele discharge are gradual. Stenoteles in heads severed from animals fed to repletion recover from inhibition of discharge in thirty minutes. The lag time between cessation of ingestion and inhibition of discharge is ten–twenty minutes.

8) A concentrated homogenate of *Artemia* injected into the gastral cavity inhibits stenotele discharge to a greater extent than does a dilute homogenate.

9) The results indicate that hydra can inhibit stenotele and desmoneme discharge, and that a metabolite(s), obtained from the *Artemia* is involved in the inhibition.

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LIMB LOSS AND THE MOLT CYCLE IN THE FRESHWATER SHRIMP, *PALAEMONETES KADIAKENSIS*

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In crustaceans, the presence of a chitinous exoskeleton imposes growth limitations on an organism. An animal must molt or shed its exoskeleton periodically in order to grow. Molt cycle control is thought to be regulated by two hormones, a molt-inhibiting hormone and a molting hormone. The X-organs are a group of neurosecretory cell bodies, located near the surface of the medulla terminalis in the crustacean eyestalk, which are presumably responsible for producing the molt-inhibiting hormone (MIH). MIH is then thought to inhibit the Y-organs, the postulated molting glands of crustaceans, from secreting the molting hormone (MH) which acts on the epidermis to initiate premolt or the preparations for ecdysis (Passano, 1960).

Another characteristic of many crustaceans is the ability to regenerate appendages which are lost. Regeneration is dependent upon molting. If a limb is removed at the beginning of the intermolt cycle, a limb bud is produced which grows during premolt (stage D of the intermolt cycle), but not until the animal molts is the limb fully formed and functional. Several molts are required to restore the limb to its original size (Goss, 1969). Since the molting process is under hormonal control, regeneration and normal growth are closely interrelated in crustaceans.

Several studies have been conducted to examine the relationship between regeneration and the molt cycle in crustaceans. Zeleny (1905) noted in three species of crustaceans: the fiddler crab, *Gelasimus* (= *Uca?*) *pugilator,* the pistol shrimp, *Alpheus* and the crayfish, *Cambarus propinquus*, that an excessive loss of limbs led to an increased rate of molting.

Loss of limbs was noted as a molt-accelerating factor for the land crab, *Gecarcinus lateralis* (Bliss, 1956, 1959). Under conditions unfavorable for molting (dry sand, air not saturated with moisture) over a 6-month period, five out of five land crabs missing 6–8 limbs completed premolt limb regeneration and molted, while only one out of nine controls missing 1–2 limbs molted. Skinner and Graham (1970) systematically examined in *Gecarcinus* the effect of loss of limbs on the initiation of molting under favorable conditions. Their results showed that the loss of numerous (6–8) limbs, both walking legs and chelae, caused the organisms to enter proecdysis almost immediately.

Similarly, several species of crabs, including the marsh crab, *Sesarma reticulatum* (Passano and Jyssum, 1963), the green crab, *Carcinus maenas,* the blue crab, *Callinectes sapidus*, and the two fiddler crabs, *Uca pugilator* and *U. pugnax* (Skinner and Graham, 1972), entered proecdysis and molted after induced multiple autotomy.

Two recent reports have mentioned unsuccessful attempts to accelerate the rate of molting by excessive limb loss. Passano and Jyssum (1963) found that the removal of six walking legs failed to stimulate several large green crabs to enter procedysis and molt. Also, the spider crab, *Libinia emarginata,* did not respond to limb loss during a ten week observation period (Skinner and Graham, 1972).

The present study was undertaken to determine how the loss of four walking legs affects the duration of the molt cycle of the freshwater shrimp, *Palaemonetes kadiakensis* Rathbun. Previous studies on limb loss have dealt primarily with Reptantia, crustaceans with heavy well-mineralized exoskeletons and adapted for crawling. Except for Zeleny's (1905) preliminary experiment with the pistol shrimp, *Alpheus,* the effect of the removal of several appendages in Natantia, crustaceans lacking well-calcified exoskeletons and adapted for swimming, has not been examined.

Also, in the literature surveyed, only the overall effect of the removal of appendages on molting was noted. Since the intermolt cycle in crustaceans can be subdivided into stages depending upon physiological and morphological changes in the organism (Drach, 1939, 1944; Passano, 1960; Stevenson, 1968, 1972; Stevenson *et al.,* 1968), this paper represents a study of the effect of limb loss at the different stages of the molt cycle.

MATERIALS AND METHODS

The freshwater shrimp used in this investigation were collected from the western end of Sandusky Bay, Lake Erie. At the start of an experiment, each organism was placed in a 4½" Carolina culture dish. The shrimp were fed daily with newly-hatched *Artemia salina* nauplii.

Five groups of organisms were established depending upon the stage (A, B, C or D) of the intermolt cycle in which legs would be amputated, with the controls set up as a separate group. All the experimental organisms were held for five consecutive molts. Between the third and fourth laboratory molts, four walking legs (3rd and 5th pairs) were amputated under 10 × magnification of a dissecting microscope. Using dissecting scissors, each leg was cut between the coxa and the basis. The control organisms were left intact for the five successive molts.

Staging the molt cycle in this investigation was determined according to the methods described by Passano (1960) for Natantia which were later modified for *Palaemonetes kadiakensis* (Thompson, 1964). Although the stages can be subdivided in some detail, only the four major stages of the intermolt cycle, A, B, C and D, were considered. Stage A is the stage immediately following ecdysis and is easily distinguished by the hollow appearance of the antennal scale and uropod setae. It was at this stage that the group A shrimp had four walking legs amputated following the third laboratory molt. Stage B occurs when the conical bases of the antennal scale spines are formed. Organisms in group B had four walking legs removed during stage B of their third laboratory intermolt cycle. Stage C occurs when 30% of the intermolt cycle has been completed and no sign of proecdysis is evident. The individuals in group C had four appendages amputated during stage C. Finally, stage D, proecdysis, which occupies 60% of the entire intermolt cycle, is subdivided quite extensively for natantians. The group D shrimp had the amputation of legs performed during late stage D, immediately prior to molt. This stage (D_4) is recognized by the new setae appearing as rods projecting into the antennal scales and uropods. The development of new setae, upon which recognition of these stages depends, could be seen under a dissecting microscope with 30 × magnification.

The laboratory room was maintained on a strict photoperiod of 14 hours of light and 10 hours of darkness which coincided approximately with the normal outdoor photoperiod. Room temperature ranged from 20° C through 26° C, with an average of 24.5° C.

RESULTS

Response to limb loss

In the freshwater shrimp, *Palaemonetes kadiakensis*, the removal of four walking legs caused an acceleration of the molting process (Fig. 1). The average time required for 50% of each group to complete the five laboratory molts (T_{50}) was: group A = 45 days, group B = 44 days, group C = 45 days, group D = 47 days, controls = 52 days. When all of the organisms subjected to limb loss had completed the four intermolt cycles ($T_{100} = 65$ days), only 92% of the controls had finished and it was not until six days later that all the controls had completed the five laboratory molts ($T_{100} = 71$ days). It was observed that on completion of the four intermolt cycles, stage A, stage B and stage C organisms had regenerated the four walking legs, but stage D organisms had not completely regained their limbs.

Response to limb loss during stage A

The amputation of four appendages immediately following a molt leads to the subsequent intermolt period being significantly shortened (Fig. 2). The 120 individuals in group A had a standard mean intermolt cycle of 13.6 days prior to limb loss. The standard mean intermolt cycle is found by taking the mean of intermolt cycles #1 and #2 ((13.4 + 13.8)/2). The mean intermolt cycle following limb loss (intermolt cycle #3) was 8.8 days which represents a decrease in intermolt cycle duration of 43.4%. The second postoperative intermolt cycle was 10.4 days. While this was longer in duration than the first, it was still reduced from the standard mean intermolt cycle by 24%.

Confidence intervals for all the means were figured with a 0.99 degree of confidence (Table I). The confidence intervals of the means for the two postoperative intermolt cycles of stage A organisms do not overlap the two preoperative intermolt cycle mean intervals, hence for a 99% degree of confidence the decrease in intermolt cycle duration following limb loss is significant.

Response to limb loss during stage B

The removal of four walking legs during stage B of the third intermolt cycle also caused a shortening of the first postoperative molt (Fig. 2). The molt cycle duration between the third and fourth laboratory molts was reduced by 27%. Also, as in group A shrimp, the second postoperative intermolt cycle was slightly longer in duration than the first postoperative intermolt cycle although it had not returned to normal. Considering that all group B shrimp had four walking legs removed on the second day following the third molt, actually 8% of the intermolt

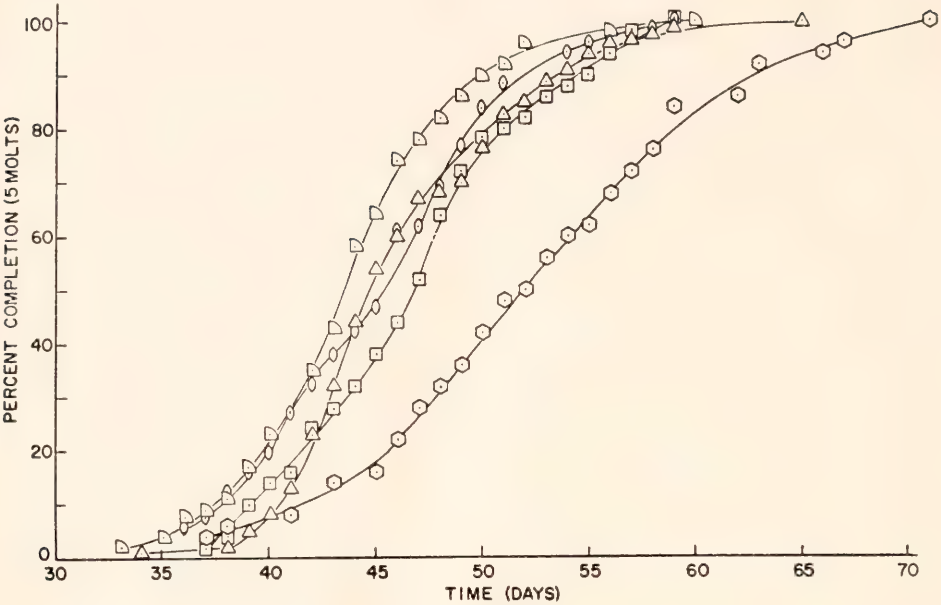

FIGURE 1. Effect of the removal of four walking legs on the duration of four successive intermolt cycles of *Palaemonetes*. All of the organisms were held for two consecutive intermolt cycles before limbs were amputated. During the third intermolt cycle four legs were removed from 120 animals which were in stage A of that intermolt cycle (open triangle), from 51 animals which were in stage B of that intermolt cycle (open arc), from 51 animals which were in stage C of that intermolt cycle (open ellipse), and from 50 animals which were in stage D of that intermolt cycle (open square). The 50 control organisms (open hexagon) were left intact for the five successive molts.

TABLE I

Effect of limb loss on molt cycle duration

Stage		A	B	C	D	X*
Number of organisms		120 (125)	51 (54)	51 (54)	50 (54)	50 (50)
Mean length (mm)		26.3	23.7	23.7	25.0	24.7
Mean intermolt cycle (days ± S.D.)						
pre-amputation	#1	13.4 ± 1.9	12.2 ± 1.9	12.0 ± 2.0	12.7 ± 1.5	12.7 ± 2.2
	#2	13.8 ± 2.3	12.9 ± 2.2	12.5 ± 2.2	12.9 ± 1.9	13.1 ± 2.2
post-amputation	#3	8.8 ± 0.9	9.2 ± 0.9	11.1 ± 1.4	13.4 ± 2.2	13.3 ± 2.4
	#4	10.4 ± 1.4	9.8 ± 1.4	9.9 ± 1.4	8.1 ± 1.1	13.6 ± 2.6
Confidence intervals for the means (days)	#1	0.5	0.7	0.7	0.5	0.8
	#2	0.5	0.8	0.7	0.7	0.8
	#3	0.2	0.3	0.5	0.8	0.9
	#4	0.3	0.5	0.5	0.4	1.0
Range (days)	#1	9–21	9–19	8–15	10–16	8–18
	#2	9–19	8–17	9–17	9–16	9–19
	#3	7–12	7–11	8–14	9–18	8–19
	#4	8–14	7–13	8–13	6–12	9–20

* Controls; brackets indicate initial number of organisms.

cycle had already been completed when limb loss occurred. Thus, the remaining intermolt cycle following amputation was 61% of a standard mean intermolt cycle.

The two postoperative intermolt cycles show significant decreases from the two preoperative intermolt cycles when the confidence intervals of the means are compared.

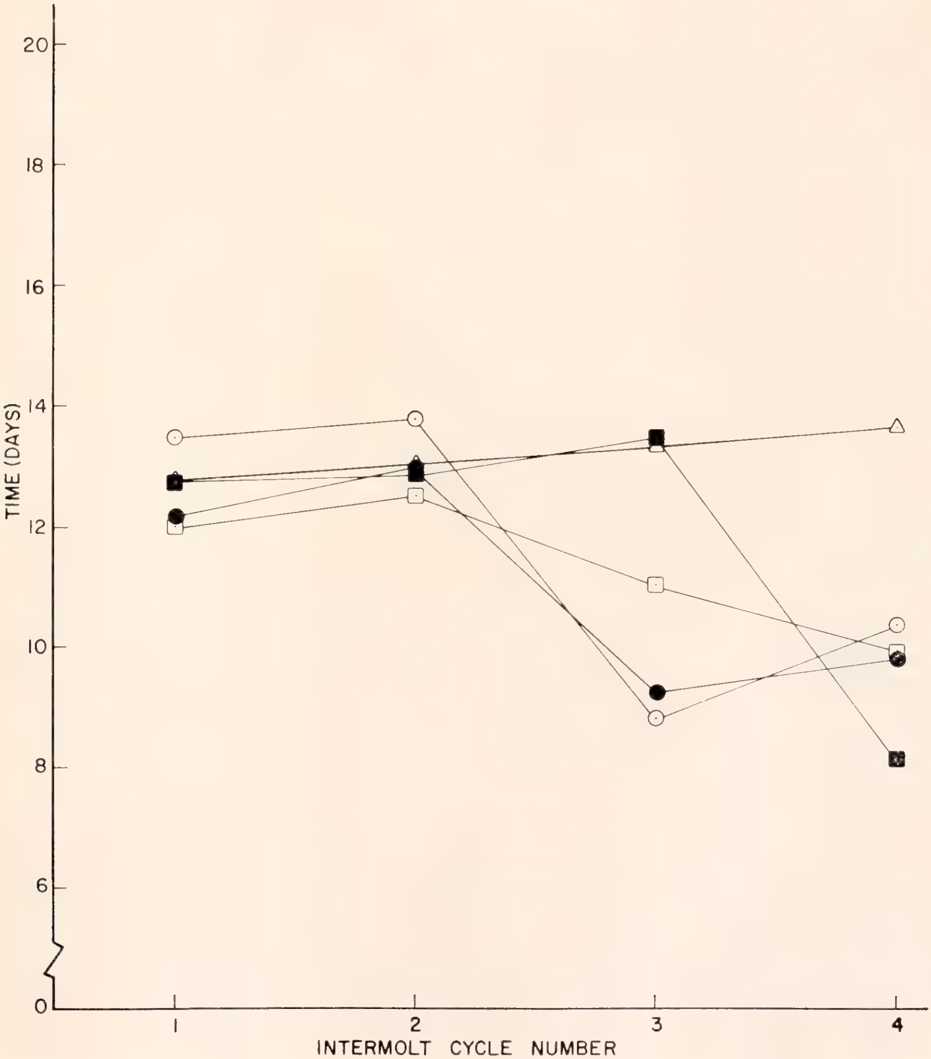

FIGURE 2. Plot of the means of the two preoperative and the two postoperative intermolt cycles of *Palaemonetes*. The group A organisms (open circle) had four walking legs removed during stage A following the third laboratory molt. The group B organisms (closed circle) had four walking legs removed during stage B of the third intermolt cycle. The group C organisms (open square) had four walking legs removed during stage C of the third intermolt cycle. The group D organisms (closed square) had four walking legs removed during stage D of the third intermolt cycle. The control organisms (open triangle) were left intact for four successive intermolt cycles.

Response to limb loss during stage C

Limb loss during stage C of the third intermolt cycle caused a shortening of two postoperative intermolt cycles (Fig. 2). The group C shrimp, which had a mean intermolt cycle of 12.2 days prior to limb loss, showed a slight reduction in the first postoperative molt (9%) and then a further reduction in the second postoperative molt (19%).

Again, if the amount of intermolt cycle completed prior to limb removal is considered, 30% of the entire intermolt cycle had passed before limbs were removed in stage C individuals and thus, the length of the intermolt cycle following amputation was actually 61% of the standard mean intermolt cycle.

As for the confidence intervals of the means, only the second postoperative intermolt cycle was a significant decrease.

Response to limb loss during stage D

When the amputation of four appendages occurred during stage D, the duration of the postoperative intermolt cycle was not affected, but the second intermolt cycle following limb loss was definitely shortened (Fig. 2). The third intermolt cycle of group D organisms showed a 5% increase over the standard mean intermolt cycle. The second postoperative intermolt cycle, however, was reduced by 37% following the removal of appendages.

The decrease of the second postoperative intermolt cycle was a significant decrease over the two preoperative intermolt cycles.

Molt cycle duration in control organisms

The control organisms, those organisms left intact and held for five consecutive molts, had four fairly consistent mean intermolt cycles (Fig. 2). Each molt cycle increased slightly over the previous one: 12.7 days to 13.1 days (+3%) to 13.3 days (+2%) to 13.6 days (+2%).

The confidence intervals of the means show that for a 0.99 degree of confidence there is not a significant difference among the four intermolt cycle durations.

Sex as a factor in limb loss

No difference was noted between males and females with respect to the response to limb loss as both sexes do show that the removal of several appendages does accelerate molting (Table II). The only factor which seems to affect the duration of the intermolt cycle between males and females is the size of the organism as females are larger (26.0 mm) than males (23.7 mm) and therefore, as shown in Table II, have mean intermolt cycles of slightly greater duration.

Mortality

The mortality rate in this investigation was 3.8% (13/337). Death following the removal of four walking legs made up only a small per cent of the total mortality (0.9%), while natural deaths (prior to the third molt) made up the largest per cent of deaths (1.8%). Other reasons for not completing the five laboratory molts included accidental deaths and severe injury resulting from ecdysis.

TABLE II

Comparison of the effect of limb loss between male and female shrimp

		Males	Females
Stage A			
Number of organisms		44	76
Mean length (mm)		24.4	27.4
Mean intermolt cycle (days)			
	#1	12.9	13.9
	#2	12.4	14.6
	#3	8.4	9.1
	#4	9.7	10.8
Stage B			
Number of organisms		26	25
Mean length (mm)		23.6	23.8
Mean intermolt cycle (days)			
	#1	12.2	12.2
	#2	12.8	13.0
	#3	8.9	9.6
	#4	9.4	10.2
Stage C			
Number of organisms		22	29
Mean length (mm)		22.5	24.7
Mean intermolt cycle (days)			
	#1	11.4	12.4
	#2	11.6	13.1
	#3	10.7	11.4
	#4	9.4	10.3
Stage D			
Number of organisms		24	26
Mean length (mm)		24.0	26.0
Mean intermolt cycle (days)			
	#1	12.5	12.9
	#2	12.2	13.5
	#3	12.7	14.1
	#4	7.6	8.5
Controls			
Number of organisms		23	27
Mean length (mm)		23.4	25.8
Mean intermolt cycle (days)			
	#1	12.3	13.2
	#2	12.7	13.3
	#3	13.1	13.5
	#4	13.0	14.2

DISCUSSION

The experimental results reported here demonstrate that the loss of four walking legs induces molting in the freshwater shrimp, *Palaemonetes kadiakensis.* They show that regardless of when limb loss occurred during the intermolt cycle, acceleration of molting ultimately resulted. These results support similar findings for several brachyuran species, including the land crab (Skinner and Graham, 1970, 1972); the marsh crab (Passano and Jyssum, 1963); and the green crab, the blue crab and two fiddler crabs (Skinner and Graham, 1972). Consequently, for at least one species of Natantia, the response to extensive limb loss is similar to that of Reptantia.

The fact that the removal of walking legs did not affect the mortality rate suggests that molts induced by extensive limb loss are normal ecdyses. Skinner and Graham's (1972) work also showed this for *Gecarcinus* with very low mortality rates following ecdysis after limb loss.

In an attempt to explain this molt-accelerating effect of limb loss, Skinner and Graham (1970) discounted the most obvious possible conclusion, that is, that a molt-inhibiting hormone is present in the walking legs. They suggested that the loss of body mass could prevent the secretion of the molt-inhibiting hormone of the X-organs. However, it was shown that the loss of tissue could not be the stimulus responsible for molt initiation since the loss of two chelipeds, which make up approximately 35% of the entire body weight, in the land crab did not accelerate molting while the loss of eight walking legs, which contribute 11–17% of the total body weight, did hasten molting (Skinner and Graham, 1972). Skinner and Graham (1972, page 230) then proposed that the "severing of a critical number of nerves" is the stimulus which induces molting.

A possible explanation of the results of the present study is that the loss of several walking legs stimulates the neurosecretory cells of the X-organs via nervous impulses to stop releasing (and also, perhaps, producing) the molt-inhibiting hormone. This would then allow the Y-organs to secrete their postulated molting hormone, and premolt, stage D of the intermolt cycle, would be initiated. In support of this hypothesis it should be noted that the length of the intermolt cycle following limb loss in three stages, A, B and C, closely approximates 60% of the previous intermolt cycle, which is considered the per cent occupied by proecdysis. The effect of limb loss on the molt cycle duration of the first postoperative intermolt cycle in group D individuals was not noted. This is to be expected since the organisms were already in stage D, proecdysis, when amputation of four walking legs occurred. However, if the duration of the second postoperative molt is compared to the previous intermolt cycle, it only amounts to 60.4% of that intermolt period suggesting that excessive limb loss stimulates the organism to enter premolt as soon as possible following the molt.

This hypothesis that stage D is initiated immediately following limb loss seems reasonable, but since molting is a cyclic event, it would seem that stages A, B and C of the intermolt cycle could not be omitted entirely. Hence, the shortening of the intermolt cycle following the removal of appendages could be the result of stages A, B and C being shortened proportionately with the animal molting in the absolute minimal time. Skinner and Graham (1972) did find that eyestalk removal caused land crabs to molt sooner than crabs stimulated to molt by limb removal. However, death usually occurred prior to or at ecdysis in eyestalkless crabs and not in crabs missing appendages. This suggests possibly that the removal of limbs, while stimulating the X-organs to stop releasing MIH, allows the change in concentration from molt-inhibiting hormone to molting hormone to be gradual enough for the organism to hasten through all the stages of the intermolt cycle prior to premolt and thus, a normal molt could occur.

Some explanation can be given for the reports which have noted that the loss of limbs has no effect on molt cycle duration. The most recent mention of inhibition of molting in crustaceans by extensive appendage loss was that the loss of all ten legs in *Gecarcinus* inhibited molting rather than accelerated it (Skinner and Graham, 1972). These crabs, which had lost their limbs in combat with other crabs, showed no sign of regeneration after 90 days in the laboratory. Starvation, which sometimes inhibits molting (Passano, 1960), was not a factor in this situation since the crabs were hand-fed.

As for multiple autotomy failing to induce molting in the green crab, *Carcinus maenas,* Passano and Jyssum (1963) indicated that these crabs were perhaps in terminal anecdysis (stage C_4T), when molting and growth in the animals have ceased. They described the crabs that they subjected to limb removal as large males and found no signs of approaching molt when they were kept in the laboratory for several months. Skinner and Graham (1972) did find that limb loss induced molting in four green crabs, but they did not mention the size of the individuals. These results suggest possibly that prior to terminal anecdysis, the removal of several appendages can stimulate molting in *Carcinus maenas,* but once the crab has ceased to molt, extensive limb loss cannot break terminal anecdysis.

Finally, no molting occurred in the spider crab, *Libinia emarginata,* following the removal of 6–8 walking legs. None of the individuals, including eyestalkless and control organisms, molted in the laboratory during the ten week observation period (Skinner and Graham, 1972). Since the organisms were held in community tanks during the experiment, it was suggested that perhaps in this organism, as in *Gecarcinus* (Bliss and Boyer, 1964), privacy at the time of molt is a "critical factor" which prevents crabs from molting in the presence of another crab. In this sense, molting would be prevented by the lack of privacy overriding any stimulus to molt from limb loss.

SUMMARY

1. The loss of four walking legs induces molting in the freshwater shrimp, *Palaemonetes kadiakensis.* This study shows that at least in one species of Natantia, excessive limb loss accelerates the molting process as has been shown in several reptantian species.

2. Removal of four appendages (two pairs of walking legs) during the four major stages of the molt cycle, A, B, C and D, produced varying results. When the appendages were removed during stage A or stage B of the intermolt cycle, the first molt following limb loss was accelerated. When limbs were removed during stage C of the molt cycle, two successive molts following limb loss were accelerated. Finally, when limb removal occurred during stage D of the intermolt cycle, the first postoperative molt was not affected, but the second postoperative intermolt cycle was shortened significantly.

3. The sex of the organism did not affect the response to the loss of numerous appendages.

4. Mortality rates due to the loss of four walking legs were negligible.

5. A hypothesis was proposed to explain this molt-accelerating effect of limb loss.

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BUDDING, SEXUAL REPRODUCTION, AND DEGENERATION IN THE COLONIAL ASCIDIAN, *SYMPLEGMA REPTANS*[1]

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Ascidians of the subfamily Botryllinae propagate asexually by pallial budding and, in some genera, by vascular budding (Berrill, 1950; Abbott, 1953; Oka and Watanabe, 1957, 1959; Milkman, 1967). Since Bancroft's pioneer work (1903), many developmental problems have been studied in botryllid ascidians, but most of the studies have been made on *Botryllus* or *Botrylloides,* so our knowledge of the budding in other synstyelid ascidians is very limited. Berrill (1940) described the mode of the budding of *Symplegma viride,* and his work is the only earlier study of budding in this genus.

Symplegma reptans is one of the common synstyelid ascidians in Japanese waters, forming thin, flat colonies. This species is easily cultured on glass plates, providing colonies that are easy to handle and to operate upon, and are clearly observable from both sides. In these points *Symplegma* is much like *Botryllus,* but it has additional features which are desirable for some types of study: (1) the zooids of *Symplegma* are "independent," each zooid possessing its own individual atrial opening; (2) the life span of each zooid is much longer than that of *Botryllus;* and (3) *Symplegma* reproduces sexually throughout the year.

Employing this species a series of descriptive and experimental studies have been carried out by the present authors. In this paper we present our observations on the life history of the species, and give special attention to the quantitative and dynamic aspects of budding. This and coming papers will show that *S. reptans* is excellent material for various kinds of study.

MATERIAL AND METHODS

For a taxonomic description of *Symplegma reptans* (Oka) see Tokioka (1949). Studies were carried out at the Usa Marine Biological Station of Kochi University, from March to May, 1969, at a sea water temperature of 15°–21° C, and at the Shimoda Marine Biological Station of the Tokyo Kyoiku University, from May to July, 1972, at a sea water temperature of 18°–24° C.

Colonies of *Symplegma* collected in the field were brought back to the laboratory with their original substrata. Here the growing margin of the colony was detached, and fragments of this cut by a razor blade were fastened by thread to glass plates and allowed to attach and grow. Oozooids were obtained by placing cultured colonies containing mature zooids in a bowl, and collecting the larvae released from them with a pipette. Then the tadpoles were settled at desired positions on a glass plate. Glass plates bearing fragments of colonies or settled larvae were

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[1] Contribution no. 234 from the Shimoda Marine Biological Station.

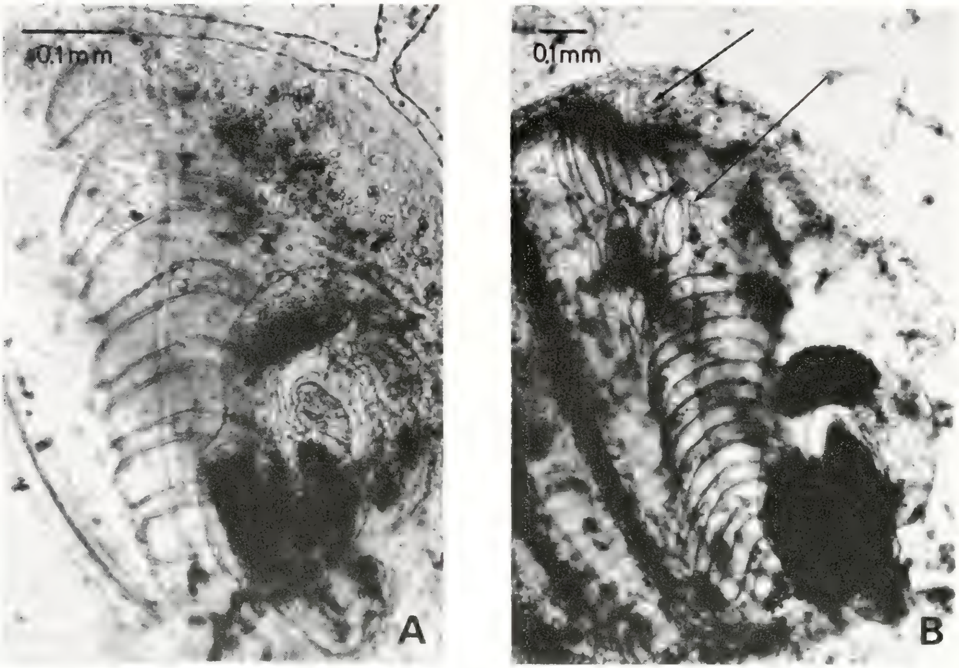

FIGURE. 1. Development of stigmata in the oozooid. (A) 5-day oozooid with 7 pairs of protostigmata, ventral view. (B) 20-day oozooid with 2 rows of stigmata and 8 protostigmata on one side of the branchial basket, ventral view.

cultured in a slidebox set in the bay. Materials were brought back daily to the laboratory, and repeated observations on *particular zooids* were made there.

For histological study the materials were fixed in cold 5% phosphate-buffered glutaraldehyde at pH 7.4 for 3 hours and embedded in Epon. The blocks were sectioned serially at 1 μ and stained with 1% toluidin blue.

OBSERVATIONS

Development, bud formation, and degeneration of the oozooid

In the laboratory most of the larvae are released during the morning hours (10–12 A.M.), and begin to metamorphose within 4–5 hours after release. At a temperature of about 15° C, heart-beat begins 2 days after attachment of larva, and 1–2 days later the apertures perforate and feeding begins. By this stage the body of the zooid is nearly transparent owing to consumption of the yolk with which the newly metamorphosed zooid is filled. The first bud is formed on the oozooid about 10 days after metamorphosis. At a temperature of 20°–23° C, the time schedule is speeded up: it takes 1–2 days after metamorphosis for perforation of the apertures, and only 3 days after metamorphosis the first bud is produced.

The oozoid continues to grow and gives off a series of 4–7 buds during its life span. In about 30 days after metamorphosis the oozooid begins to degenerate. The time of degeneration is little affected by temperature. Colonies grow faster under warmer conditions, so the size of the colony at the time degeneration of the oozoid occurs varies with the temperature. Degeneration occurs more or less suddenly. It is not preceded by a decrease in the growth rate or by a slowdown of the heart-beat, both of which are observed in the degeneration of blastozooids.

A young functional oozooid has 7–9 protostigmata on each side of the pharynx (Fig. 1A). The anterior 3–4 protostigmata are subdivided successively from anterior to posterior during the growth of the oozooid (Fig. 1B. By the time degeneration occurs the subdivision of the anterior 3–4 pairs is completed, but the posterior 3–5 ones remain undivided. The oozooid thus has at least 3 pairs of protostigmata throughout its life.

The blastozooids produced by successive buddings of an oozooid spread out radially and surround the oozooid. These zooids in turn produce buds, so at the time of degeneration the oozooid is situated at the center of the colony composed of 70–80 zooids at various stages of development.

No gonad is formed in the oozooid before it degenerates. Even in oozooids which were surrounded by blastozooids with visible gonads no sign of gonad formation was observed.

Development and bud formation of the blastozooid

As stated above, the first bud, or first blastozooid, of an oozooid is formed about 10 days after metamorphosis at about 15° C. The bud arises from a definite site on the antero-ventral wall of the right atrial chamber. This site is very close to the base of a test vessel (Fig. 2A). The second and the subsequent buds are also formed in the same region. The epidermis and atrial epithelium of this region thicken and form a double-layered hemispheric vesicle about 50 μ in diameter at this stage. With further development the bud becomes nearly spherical. The inner layer of the bud then detaches from the parental atrial epithelium whereas the outer layer remains continuous with the parent wall by a broad stalk through which the blood flows (Fig. 2B). Within 24 hours after the formation of a hemisphere vesicle the stalk is broken. However, a new test vessel arises from the anterior wall of the bud (Fig. 2C); this fuses with a test vessel of the parent, so the developing bud is again connected with the parental circulatory system.

The developing bud, or blastozooid, forms its own first bud at a very early stage of development, when it is only 3–4 days old (17–20° C) and is not yet functional (Fig. 2D). At this stage the developing zooid is about 0.3 mm in length and 0.26 mm in width. The heart becomes functional when the developing zooid is 6–7 days old (17°–20° C) and has reached an average size of 0.39 × 0.29 mm (Fig. 2E). One day after the heart begins to beat the apertures become perforated (Fig. 2F). After this the zooids feed actively and grow quickly (Fig .3).

Each developing zooid gives rise to a series of 3–6 buds during the period of development and growth (Fig. 4). The budding phase of a blastozooid comes to an end at about the time a pair of gonads appear clearly. At this stage the zooid measures about 1.7 mm in length, and 1.3 mm in width. The oocyte of this stage is about 35 μ in diameter. As a rule, no bud formation occurs after this stage, and the gonad develops quickly. In other words, the budding, or growing phase gives place to the sexual reproduction phase. It takes, on an average,

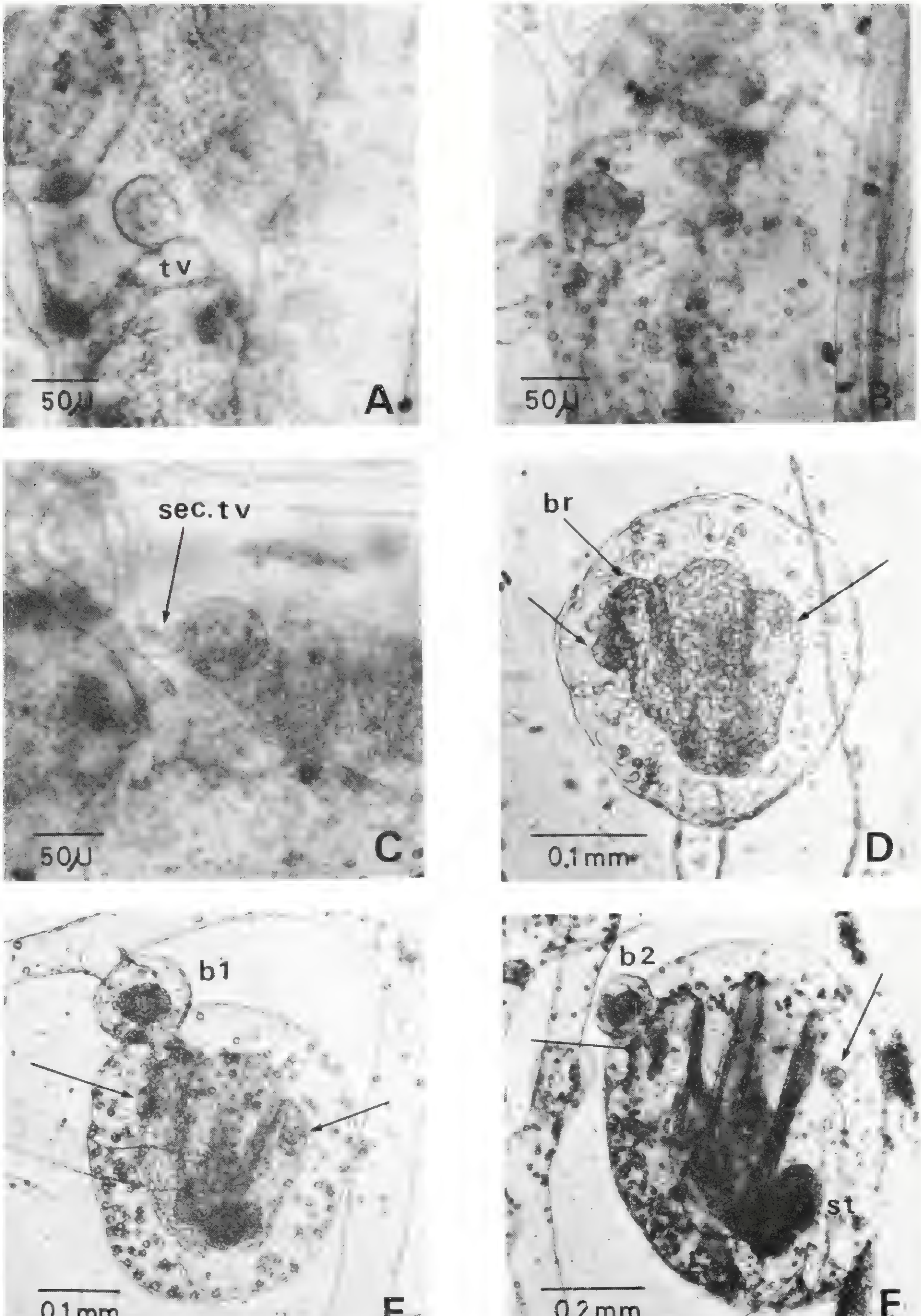
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FIGURE 2. Development of a bud arising from an oozooid. (A) Appearance of bud vesicle. (B) Bud vesicle with a broad test vessel. (C) 1-day bud with a secondary test vessel. (D)

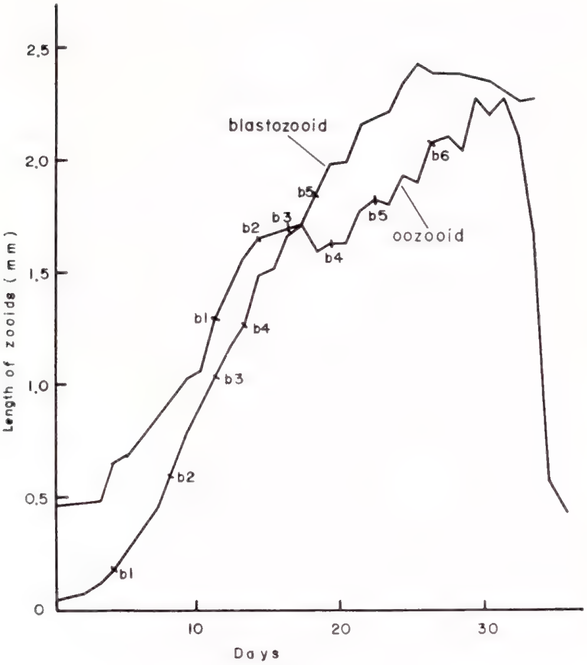

FIGURE 3. Growth of an average oozooid and an average blastozooid. Abbreviations are the same as Figure 2.

14.6 days (18°–24° C) to reach this stage after the "birth" of a blastozooid as a bud rudiment (Figs. 5, 6).

The buds formed on the wall of a developing blastozooid develop in the same manner as the buds formed on an oozooid. No significant differences have been found between the time schedule of development of buds derived from an oozooid and that of buds derived from a blastozooid. Blastozooids derived from blastozooids also produce a succession of 3–6 buds during their budding phase. They stop bud formation on an average of 12.8 days after their birth, and enter the sexual phase.

Buds and young zooids spread out to the periphery of the colony, and older zooids remain in the central region. A large colony thus consists of two kinds of zooids with respect to reproductive activity; zooids in sexual phase which occupy the central region of the colony and zooids in the asexual phase occupying the periphery.

The intervals of the successive buddings in each oozooid and in each blastozooid are summarized in Table I. At about 17° C, the first budding of the oozooid takes place later than at about 20° C. However, the intervals between successive

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3-day bud in which the first bud of the next generation is being formed. (E) 6-day bud in which weak heart-beat is seen. (F) 8-day blastozooid which has begun feeding. Arrows indicate gonadial rudiment; b1 (b2), first (second) bud; br, thickening of peribranchial wall as a bud rudiment; sec. tv, secondary test vessel; st, stomach; tv, test vessel.

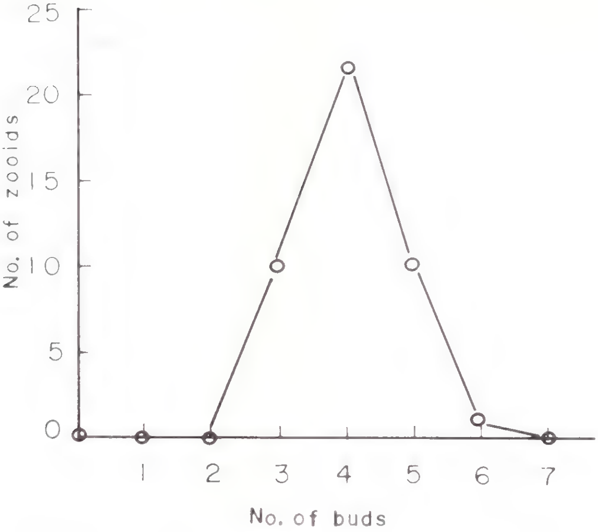


FIGURE 4. Variation in the number of buds produced per blastozooid.

buddings in oozooids at 20° C is almost same as that in blastozooids at 20° C; in both, budding occurs every 2–3 days (Fig. 7). The 6th and 7th buddings tend to occur after a longer interval than earlier ones.

The blastozooids surrounding an oozooid sometimes close their budding phase before the oozooid begins to degenerate. The buds produced by old oozooids and aged blastozooids tend to become abortive.

Sexual reproduction and degeneration of the blastozooid

The oozooid reproduces only asexually during its life span. The blastozooid reproduces asexually until the gonads have developed to the stage where a pair of

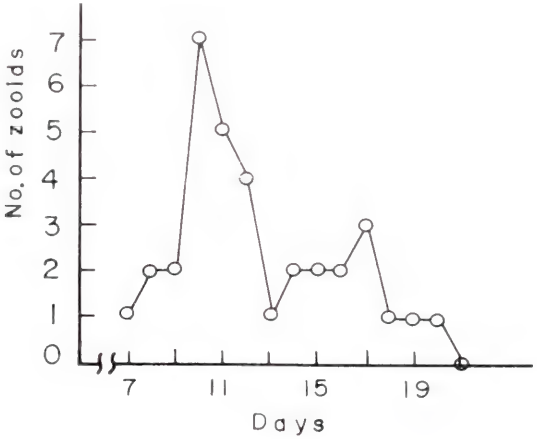


FIGURE 5. Age in days of blastozooids at the time of formation of the last bud.

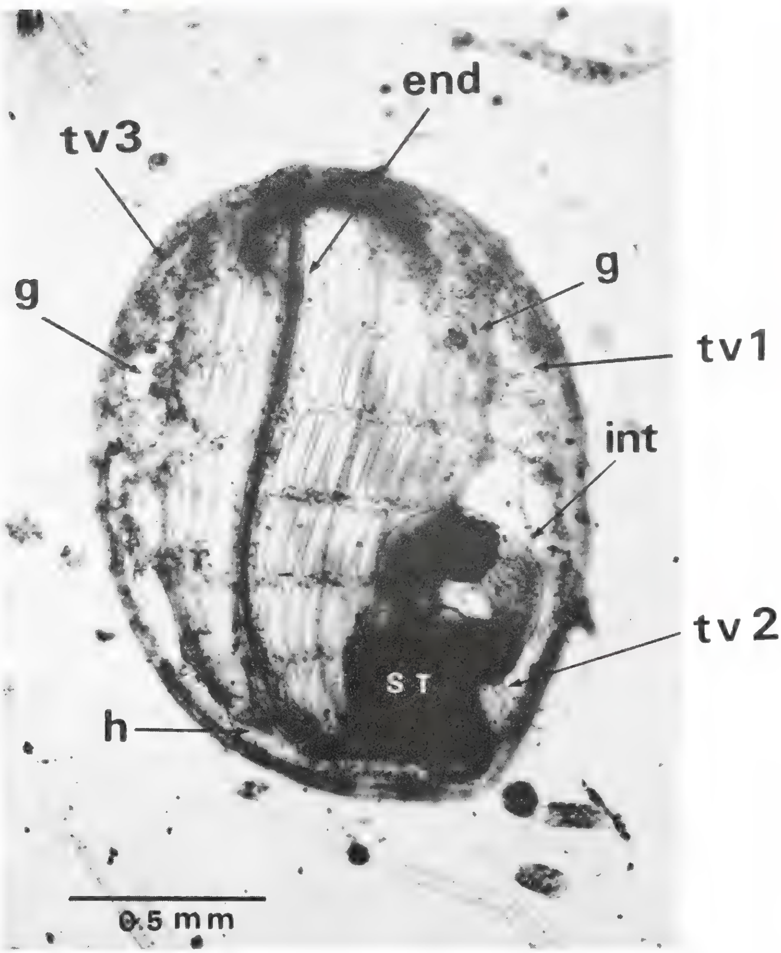


FIGURE 6. The 13-day blastozooid which has just stopped bud formation; end, endostyle; g, gonad; h, heart; int, intestine; st, stomach; tv(1–3), test vessel.

TABLE I

Average intervals between two successive buddings in each zooid (15°–21°C)

	Oozooid	Blastozooid
Metamorphosis to 1st budding	10.8 (days)	
Blastozooid "birth" to 1st budding		4.1 (days)
1st–2nd budding	2.3	2.2
2nd–3rd budding	2.5	2.2
3rd–4th budding	4.0	2.5
4th–5th budding	2.8	3.0
5th–6th budding	4.0	4.0
6th–7th budding	8.0	

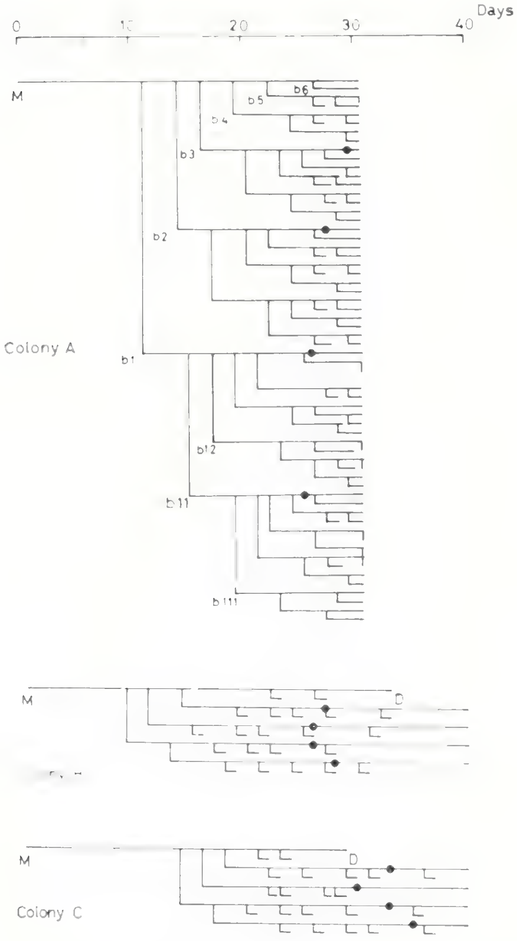
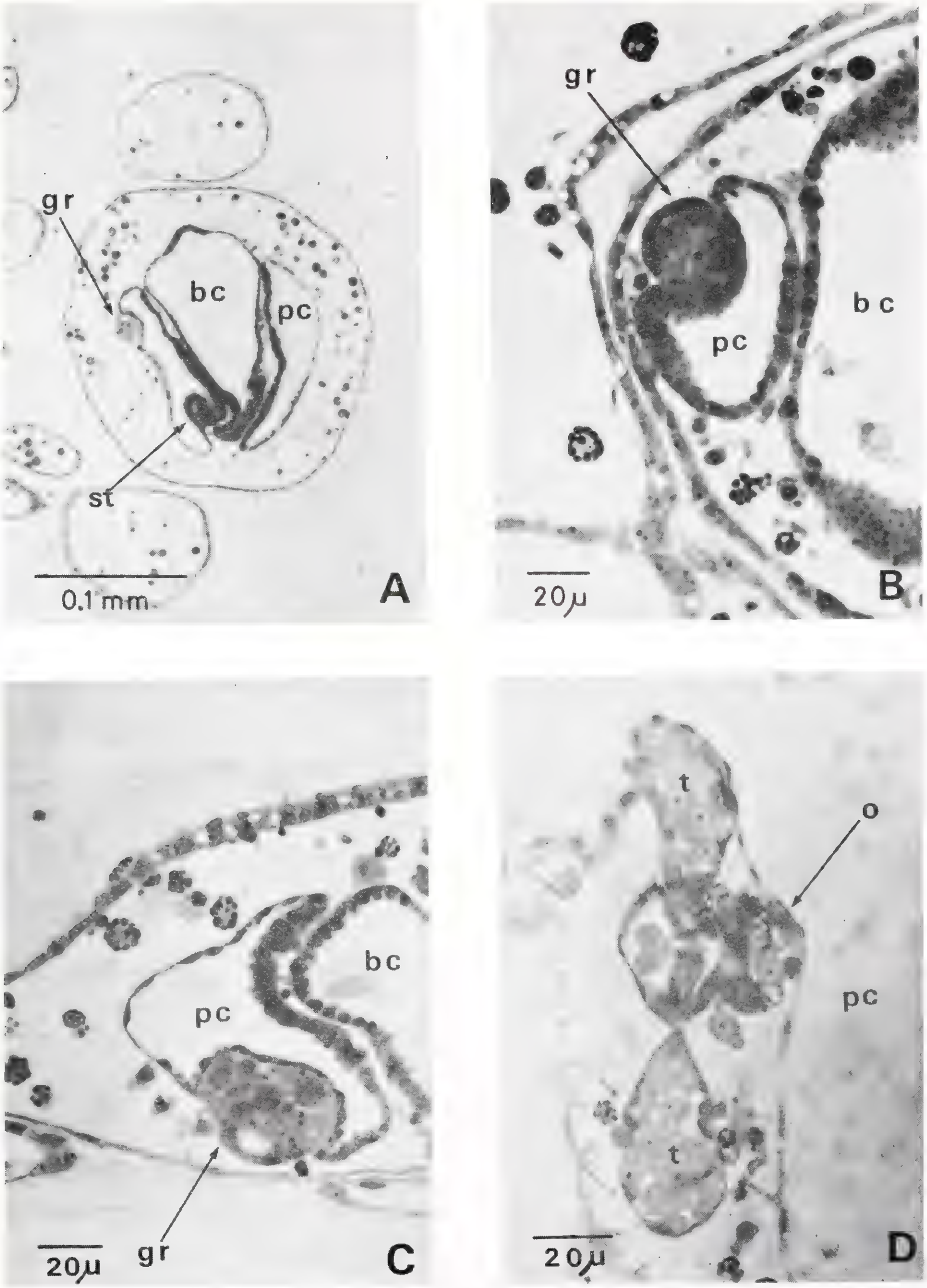


FIGURE 7. Genealogies of three colonies originating from oozooids. Nomenclature of the zooids is taken from Berrill (1940). The end of lines indicates the end of observation of the zooids; D, degeneration of the zooid; M, metamorphosis; solid circle, appearance of gonads.

testicular follicles and an ovarian follicle are clearly observable under a microscope in living materials.

The sexual phase starts with this stage, but the gonad rudiment appears much earlier. It appears as a mass of lymphocytic cells in the genital tracts around the time when the first bud is formed in the developing blastozooid (Fig. 8A). In living materials so many blood cells are found in the genital tracts that it is impossible to identify the gonadial rudiment, but in fixed material identification is easier. The gonadial rudiment grows as the zooid grows (Figs. 8B–8D). As shown in Figures 9A–9D, the eldest oocyte enters to quick-growing phase about 20 days (18–24 C) after the birth of the blastozooid. In about 5 days the growing oocyte reaches its maximum size, 220 μ in diameter.


gr
bc
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0.1 mm
A
gr
bc
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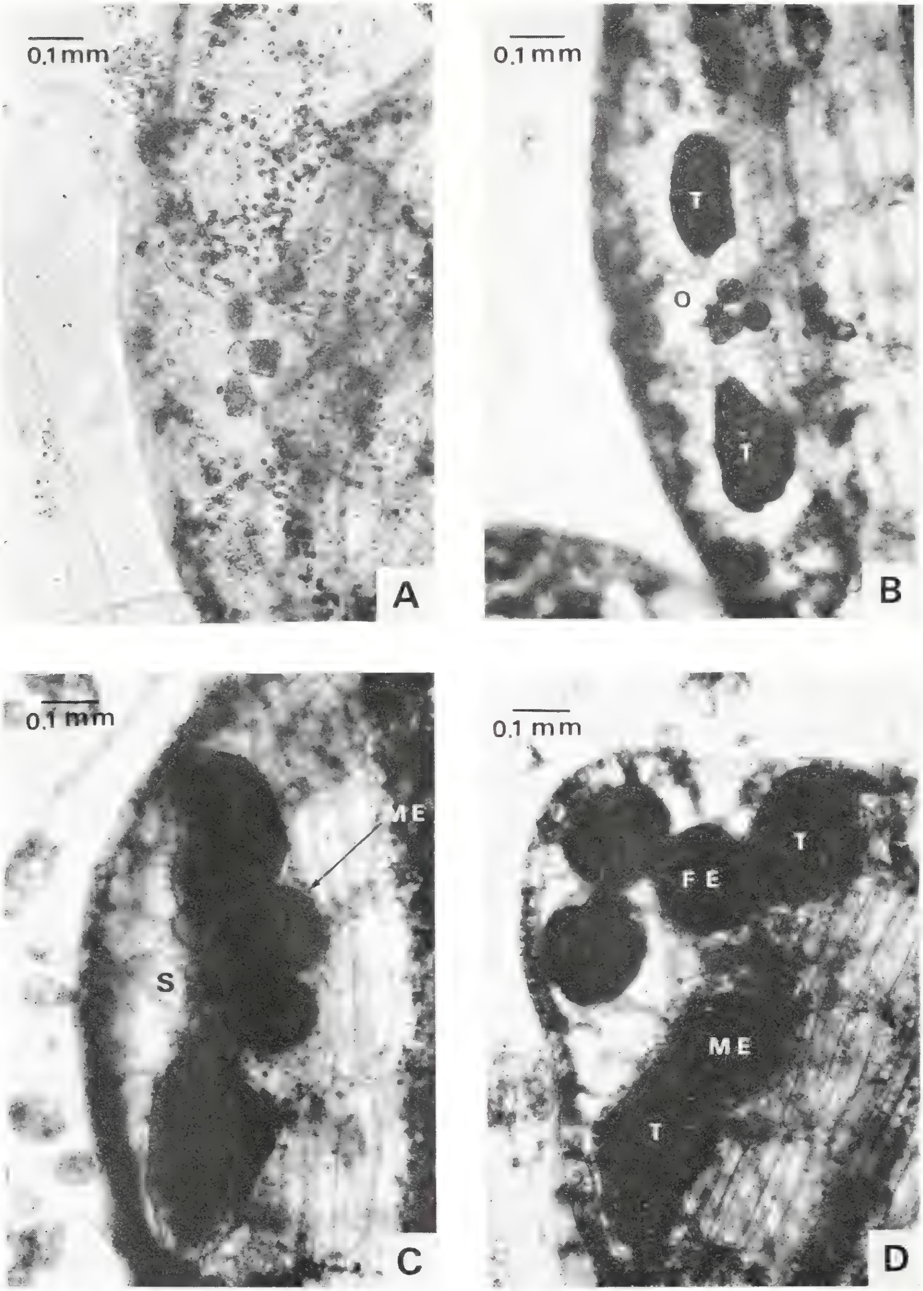
t
20μ
D

FIGURE 8. Formation of the gonadial rudiment by an aggregation of lymphocytic cells; (A) frontal section of a 4-day blastozooid, showing the position of the gonadial rudiment; (B) gonadial rudiment of a 4-day blastozooid; (C) longitudinal section of a 5-day blastozooid; (D) gonad of an 11-day blastozooid; bc, branchial cavity; gr, gonadial rudiment; o, ovary; pc, peribranchial cavity; st, stomach; t, testis.


0.1 mm
A
0.1 mm
T
O
T
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ME
S
C
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T
FE
ME
T
D

FIGURE 9. Development of the gonad; (A) 11-day zooid; (B) 15-day zooid; (C) 24-day zooid; (D) 28-day zooid; FE, fertilized egg; ME, mature egg, O, ovary; S, brood sac; T, testis.

With enlargement of the oocytes, the shape of the zooid changes from ellipsoidal to triangular. In the anterior region on both sides of the body the epidermal and peribranchial wall protrude laterally, and the anterior part of each peribranchial cavity expands laterally. A brood sac derived from the ovary protrudes into each expanded cavity (Fig. 10A). After the eggs have reached maximum size they are ovulated in the brood sac where fertilization occurs and development of the tadpole larvae takes place.

In the early stage of gonad development, 6–7 oocytes are seen in the ovary. Before these oocytes reach their maximum size, new young oocytes appear. Further oocytes also appear as the enlarged ova are ovulated. So, in the aged zooid, 5–7 oocytes of various stages and about 3 ova are seen in the ovary. Sometimes, the larva developing in a brood sac moves the tail actively. At a temperature of 18°–24° C, it takes 30 days from the birth of the blastozooid, and 5 days from fertilization, to the liberation of the first larva.

Some of the blastozooids have been observed to degenerate in 60–70 days after their birth. In these zooids the internal organs except for the heart begin to disintegrate, and the zooids become transparent (Fig. 10B). These zooids contain a few larvae or ripening eggs, but before degeneration is complete all the larvae are released. From the number of cases oberved we cannot say that the life span of the blastozooid is limited to 60–70 days, but it is clear that blastozooids live for 60 days or more, and that at least some of them degenerate before the death or degeneration of the whole "colony." Time schedules of development for an average oozooid and an average blastozooid are shown in Figure 11.

DISCUSSION

While the mode of budding of *S. reptans* is shown to be fundamentally identical with that of *S. viride* as reported by Berrill (1940), several new facts are revealed by the present study. The time schedule of budding and degeneration of zooids were previously unknown, and the development of the buds arising from the oozooid was "described only partially" by Berrill (1940). Repeated observations on particular oozooids in the present study reveals that the oozooid makes its first

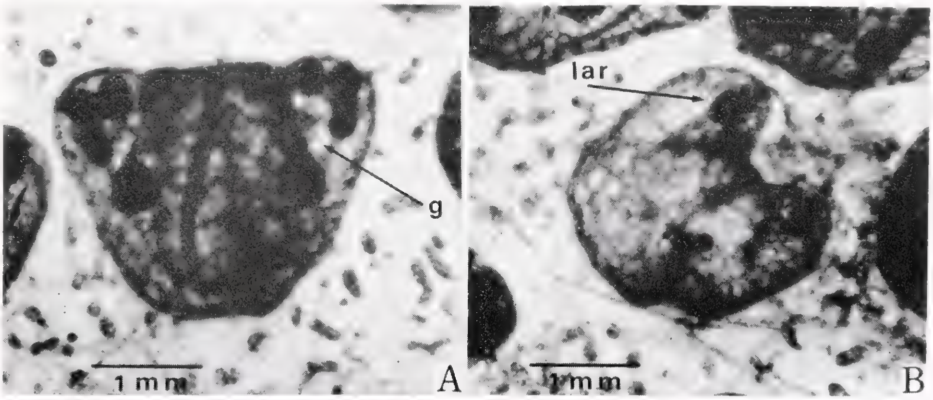

FIGURE 10. (A) Fully mature 35-day blastozooid with larvae; (B) Degenerating 65-day blastozooid in which all organs but the heart have disintegrated; g, gonad; lar, larva.

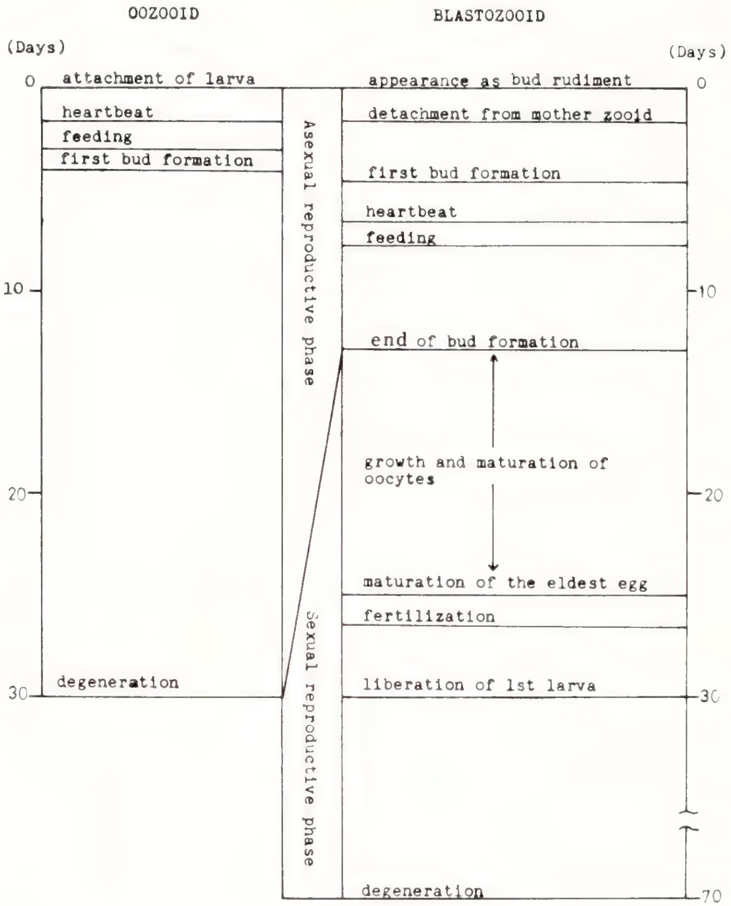
OOZOOID
BLASTOZOOID
(Days)
(Days)
0
attachment of larva
heartbeat
feeding
first bud formation
10
20
30
degeneration
Asexual reproductive phase
Sexual reproductive phase
appearance as bud rudiment
detachment from mother zooid
first bud formation
heartbeat
feeding
end of bud formation
growth and maturation of oocytes
maturation of the eldest egg
fertilization
liberation of 1st larva
degeneration
0
10
20
30
70

FIGURE 11. Time schedules of development in average oozooids and blastozooids at about 20° C.

bud about 10 days (17°–20° C) after metamorphosis and thenceforth it continues to produce buds at intervals of 2–8 days until it degenerates. The oozooid produces 4–7 buds during its life span of about 30 days.

It is also shown that in oozooids neither complete subdivision of the protostigmata nor gonad formation takes place before degeneration begins, so the oozooid is distinguishable from the blastozooids in the same colony by these morphological characters. It is of interest that, likewise, no gonad is formed in the oozooid of the synstyelid *Metandrocarpa taylori* (Watanabe, 1970) or in that of the pelagic tunicate *Doliolum* (Berrill, 1950).

As to the number of the buds produced by each blastozooid, Berrill (1940) states that in *S. viride*, "As far as can be determined, this (fourth bud) represents the last of the bud series." The present study shows that in *S. reptans* the fourth bud is not always the last bud, and that the number of buds produced by a developing blastozooid varies from 3 to 6.

In *S. reptans* the budding phase of a blastozooid comes to an end about the time when a pair of gonads becomes clearly visible, and as a rule no budding takes place in mature zooids. However, the rudiment of gonad is shown to be formed early in the asexual phase, so here the sexual phase is not so clearly segregated from the asexual one as in *Hydra* (Loomis and Lenhoff, 1956; Loomis, 1959) or in the hydroid *Podocoryne* (Braverman, 1962).

At an early stage of gonad formation the lymphocytes were found to aggregate in the genital tracts. The same phenomenon was seen in *Distomas variolosus* (Newberry, 1968) and in *Botryllus primigenus* (Mukai and Watanabe, 1972). These observations suggest that lymphocytes are usually involved in gonad formation in synstyelid ascidians. One of the most important problems is whether or not these lymphocytes are on the so-called germ cell line. Growing oocytes at various stages have been observed in the common blood system of *Botryllus* (Berrill, 1941; Izzard, 1968; Mukai and Watanabe, 1972), but such oocytes are not found in the test vessels of *S. reptans.* As far as the microscopical sections show, germ cells seem to arise directly from the mass of the lymphocytes. The lymphocytes of ascidians are also known to play an important role in the budding of *Botryllus* (Oka and Watanabe, 1957; Milkman, 1967), *Botrylloides* (Oka and Watanabe, 1959), and *Perophora* (Freeman, 1964). These facts call for further studies on the developmental capacities of ascidian lymphocytes. It would also be desirable to trace the origin of the lymphocytes found in the genital tracts, to see whether the germ cells of ascidians are determined in an early stage of development as in mammals and insects.

As to the life spans of the zooids of *Symplegma,* Berrill (1940) states, "unlike *Botryllus,* the constituent zooids of a colony of *Symplegma* persist while the colony lives and are not brief transients." Our observations show that oozooids of *S. reptans* degenerate abut 30 days after metamorphosis, while the blastozooids of the same colony remain healthy. The oozooid of *Aplidium californicum* also degenerates in 23–32 days after metamorphosis, whereas all the blastozooids of the same colony are very active (Nakauchi, unpublished). The factors governing the degeneration of particular individuals in a *Symplegma* colony are being investigated.

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SUMMARY

The life history of *Symplegma reptans* was studied by repeated observations on particular living animals and by observations on sectioned materials.

The oozooid produces its first bud about 10 days (17°–20° C) after metamorphosis, and produces a series of 4–7 buds by the time the oozooid begins to degenerate. The degeneration occurs about 30 days after metamorphosis. No

gonad is formed in the oozooid even at the time when gonads are seen in the blastozooids of the same colony. Of the 7–9 protostigmata present in the newly metamorphosed oozooid, the anterior 3–4 are subdivided by the time of degeneration, but the posterior 3–5 remain undivided throughout the life of the oozooid.

A blastozooid makes its own first bud 3–4 days (17°–20° C) after its first appearance, when it is still scarcely more than "bud" itself. The growing blastozooid makes a succession of 3–6 buds at intervals of 2–4 days. Bud formation by a blastozooid come to an end about the time its gonad become macroscopically visible. Blastozooids live for 60 days or more, but at least some of them degenerate before the death or degeneration of the colony.

Microscopical sections suggest that the germ cells are formed directly from lymphocytes.

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FOOD-RESOURCE PARTITIONING IN THE DEPOSIT FEEDING POLYCHAETE *PECTINARIA GOULDII*

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Although many benthic marine environments often support large populations of deposit feeding invertebrates, few studies have examined food-resource partitioning in this group of animals. Most studies dealing with different modes of feeding concentrate primarily upon the morphological characteristics of feeding structure (see Yonge, 1928). Sanders, Goudsmit, Mills and Hampson's (1962) study of the deposit feeders associated with the intertidal sand flats of Barnstable Harbor, Massachusetts, was one of the first attempts to categorize different types of material actually being ingested by detritovores. One of their major conclusions from the examination of the gut contents of 36 species of invertebrates was that most species exhibited generalistic feeding habits. Other studies (*e.g.*, Whitlatch, 1972) have shown some deposit feeders to be highly selective in their choice of particles.

The importance of deposit feeders in marine benthic communities is demonstrated by studies dealing with their effects on sediment stability and trophic structure (Rhoads and Young, 1970), distributions of other benthic organisms (Rhoads and Young, 1971) and water turbidity and nutrient recycling (Rhoads, 1973). Gordon (1966) has shown that *Pectinaria gouldii* (Verrill, 1873), a deposit feeding polychaete, has an annual sediment working rate of about 600 gm of sediment/worm. Using averages of 10 and 40 worms/m^2, he concluded that the sediment of one square meter and 6 cm deep could be completely reworked in 15 and 6 years, respectively. Because of the high rates of sediment working, this polychaete could have many effects upon the benthic environment. In the present study, *P. gouldii* was examined to ascertain feeding selectivity and the resultant effects upon the benthic community.

METHODS

Specimens of *P. gouldii* were collected at low tide from Little Sippewisset salt marsh, a shallow tidal enbayment located on the eastern shore of Buzzards Bay, north of Woods Hole, Cape Cod, Massachusetts (41°34′30″, 70°38′20″). The exact position of individual worms in the flat was determined by gently sliding a hand across the sediment; the tubes of the worms could easily be located in this manner. Twenty centimeter diameter cores were then taken and the sediment was removed with the worms in place. The sediment cores were brought to the laboratory and cooled at 4° C until time of dissection. The cores were vertically dissected (always within two hours of collection), and the worms were removed and immediately frozen. A sediment sample was taken at the level of the mouth and preserved in 70% alcohol.

The sediment sampled near the mouths of the worms was analyzed to determine particle size, percentage particle type abundance, and percentage organic material available to the organisms. Two subsamples of the sediment were stained with histological stains. Mercuric bromphenol blue was used for staining protein-containing material (Mazia, Brewer and Alfert, 1953). Humason (1967) states that this method appears to stain most proteins and peptides. Periodic acid Schiff reagent was used for staining carbohydrates. Humason (1967) mentions that this technique will stain a variety of substances including most protein-carbohydrate complexes. Sudan black B used for staining lipid material (Humason, 1967) was discontinued after preliminary staining of the sediment showed no reaction. The stained sediment was mounted on a slide and twenty random microscopic fields were recorded to determine the percentage of stained and unstained material. The particle size distribution of the samples was obtained by measuring the first 300 particles encountered on the slide.

Individuals of *P. gouldii* were removed from their tubes and sediment was collected from the foregut (as near the mouth as possible) and the intestine (after the digestive gland) with a modified Pasteur pipette. After the gut fractions were washed several times in distilled water to remove mucus, they were preserved in 70% alcohol. The procedures outlined above for the sediment fractions were employed to determine abundance of particle types and size distributions for these samples. The sediment and gut contents were examined at 200 × using a bright-field microscope with a calibrated eye-piece micrometer.

The results of the analysis of abundance of different particle types are expressed as percentage particle abundance. This measure is more important than volumetric and weight estimates in feeding studies since it reflects the relative amount of different types of particles an organism encounters while feeding. The measure, therefore, is valuable in determining food selection of different fractions of the environmental food-complex by the organisms.

The statistic used to determine feeding selectivity is described by Ivlev (1961) and is calculated as $E' = (r_i - p_i)/(r_i + p_i)$. For the ith food type, r_i equals the percentage ingested by the predator and p_i is the percentage of that food type available in the environment. The coefficient is finitely bounded and evenly distributed about zero. Zero indicates non-selective feeding; values from -1 to 0 indicate avoidance; and values of 0 to $+1$ indicate feeding preference.

Additional worms were brought to the laboratory for studies dealing with feeding habits. Fresh worms and sediment were collected and placed into 50 ml beakers. Observations of the feeding behavior were recorded using a vertically mounted dissecting microscope.

RESULTS

Feeding habits

P. gouldii, which lives in a tapered tube composed of a single layer of large mineral grains, is normally positioned obliquely below the surface of the sediment. The head of the worm, located at the lower and larger end of the tube, is 1–5 cm

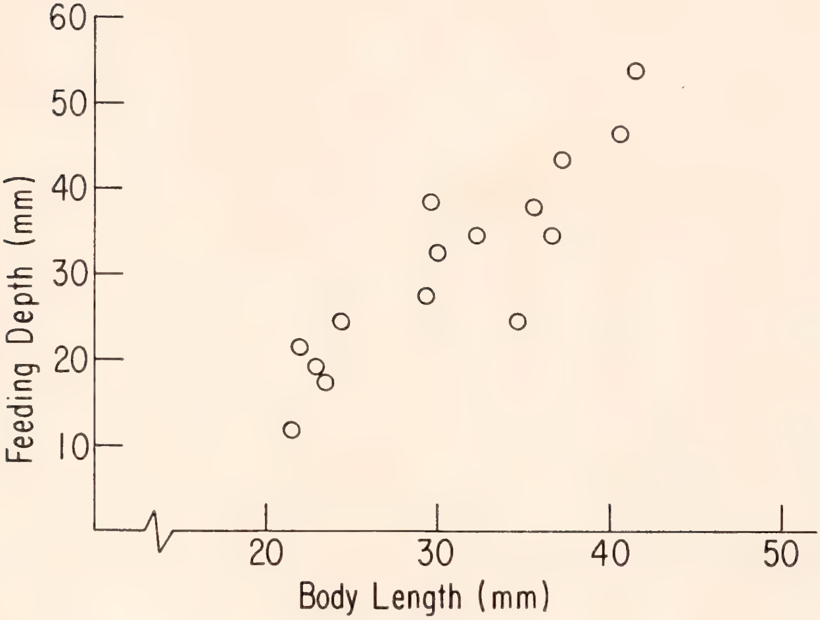

FIGURE 1. Relationship between body length of *Pectinaria gouldii* and depth of feeding.

below the surface. A highly significant relationship ($r = 0.90$, $P < 0.05$) exists between the length of *P. gouldii* and the depth at which the worm feeds (Fig. 1). Smaller individuals are found feeding closer to the surface than larger individuals.

P. gouldii has long golden paleae used strictly for digging and numerous long, ciliated, grooved tentacles which bring sediment to the mouth. When feeding, the tentacles independently select particles from all areas near the mouth. The selected particles are brought to the mouth and rejected particles fall to the bottom of a feeding cavern which the worm creates while feeding. The selected sediment is either ingested or transported between the worm's body and tube to the surface of the flat where it is deposited around the posterior end of the tube. As the worm slowly moves through the sediment, the small excavated cavity continually collapses and fills in with sediment from the sides. Previous ecological studies on the genus *Pectinaria* include Gordon's (1966) work on the sediment reworking activities of *P. gouldii* and Waston's (1927) description of the natural history of *P. koreni*, a European species.

Particle composition of the environmental food-complex

The particle composition of the sediment obtained near the mouths of fifteen specimens of *P. gouldii* is presented in Table I. The classification of different possible food sources available to the polychaete are as follows: (a) *Encrusted minerals*—mineral grains encrusted with living matter, organic debris, or other encrustations. Encrusted material was determined by visual observations. Steele and Baird (1968) have found that nearly all of the organic component in a sandy

TABLE I

Per cent particle type abundance of sediment sampled near the mouth of Pectinaria gouldii

Particle type	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
Mineral $>75\ \mu$ encrusted	3.8	6.3	13.4	13.2	9.2	7.5	7.7	1.3	3.7	0.6	0.9	5.9	4.4	6.7	7.0
Mineral $>75\ \mu$ not encrusted	5.6	8.7	28.9	16.0	12.3	5.6	13.5	2.1	1.8	1.4	2.3	9.8	6.7	9.4	13.9
Mineral 25–75 μ encrusted	0.0	0.0	0.5	0.0	0.8	0.0	0.0	0.3	0.5	0.2	0.1	0.4	0.2	0.1	0.4
Mineral 25–75 μ not encrusted	9.3	10.8	6.7	8.3	7.9	7.6	8.6	6.2	7.4	5.7	6.1	14.0	12.4	9.7	10.1
Mineral $<25\ \mu$	44.6	41.9	32.5	44.1	41.9	53.4	50.4	52.5	50.7	53.9	49.9	46.2	45.8	49.7	41.9
Floc aggregates	31.5	27.0	16.6	14.0	19.3	21.3	15.9	30.2	32.8	33.3	31.5	18.8	25.7	19.5	22.4
Fecal fragments	3.0	3.2	1.1	1.8	0.6	2.9	2.3	2.2	1.7	1.8	3.9	3.7	3.3	0.8	2.5
Plant fragments	0.9	0.7	0.0	0.3	0.4	0.5	0.0	0.5	0.2	0.6	0.7	0.3	0.5	0.5	0.8
Dead diatoms	0.8	0.8	0.3	1.8	0.8	0.9	1.5	2.5	0.3	1.7	2.7	0.4	0.4	1.0	0.9
Others	0.5	0.6	0.0	0.5	1.6	0.3	0.1	2.2	0.9	0.8	1.9	0.5	0.6	2.6	0.1

beach existed as encrustations upon sand grains. This includes bacteria and other micro-organisms described by Anderson and Meadows (1969) and Batoosingh and Anthony (1971). It seems likely, therefore, that encrusted mineral grains could be an important food source for deposit feeding organisms. (*b*) *Fecal material*—fecal pellets and fragments found in the sediment. The importance of fecal material as a food source for invertebrates has been demonstrated by Newell (1965) and Johannes and Satomi (1966). (c) *Floc aggregates*—amorphous material consisting primarily of organic and inorganic debris. The origins of this material may be from both plant and animal sources and is usually referred to as detritus. Considerable research has been directed in defining detritus as a food stuff (Darnell, 1967; Fox, 1950; Odum and de la Cruz, 1967; Adams and Angelovic, 1970). (d) *Plant fragments*—dead and decomposing plant material. Odum and de la Cruz (1967) have shown that decomposing plant material is actually a better food source than living tissue.

The composition of the different particle types associated with the sediment is quite consistent between samples (Table I). Mineral grains less than 25 μ are the most abundant particle type. Mineral grains and floc aggregates comprise more than 90% of the total sediment.

Feeding selectivity

From his study of sediment working of *P. gouldii* collected from Barnstable Harbor, Gordon (1966) concluded that the polychaete was a non-selective deposit feeder and did not sort particles for ingestion according to size. His conclusions were based upon comparisons of particle size distributions in the gut to that of sediment collected on the surface of the sand flat where the worms were feeding. Gordon failed, however, to note the length of individual worms or sample the sediment that the worms were directly feeding upon.

In the present study, *P. gouldii* is found to be selectively feeding upon the sediment at Little Sippewisset salt marsh. Figure 2 shows that larger worms are selecting, on the average, larger particles than smaller worms ($r = 0.85$, $P < 0.05$). This may be the result of two factors. Smaller individuals of *P. gouldii* feed closer to the surface than larger individuals. Since the average

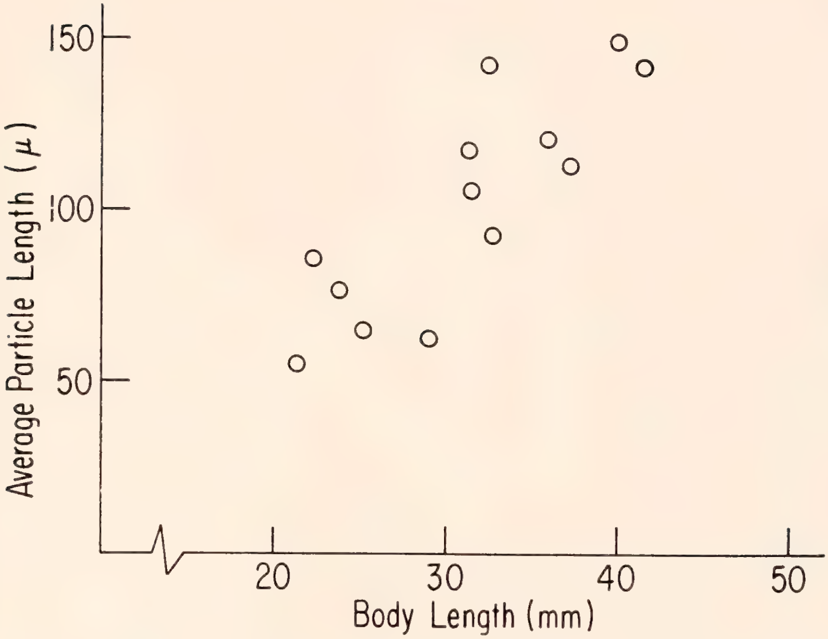

FIGURE 2. Relationship between body length of *Pectinaria gouldii* and length of average-sized particle ingested by the polychaete.

particle size of the sediment increases slightly with depth, the animals may be feeding in completely different sedimentary environments. Secondly, the size selection may be the result of a morphological constraint placed upon the organisms (*e.g.*, mouth size, ability of the feeding tentacles to obtain particles, *etc.*) that changes with size (age) of the polychaete.

Feeding selectivity coefficients are presented in Figure 3 for the six most abundant particle types found in the foregut (more than 95% of the particles

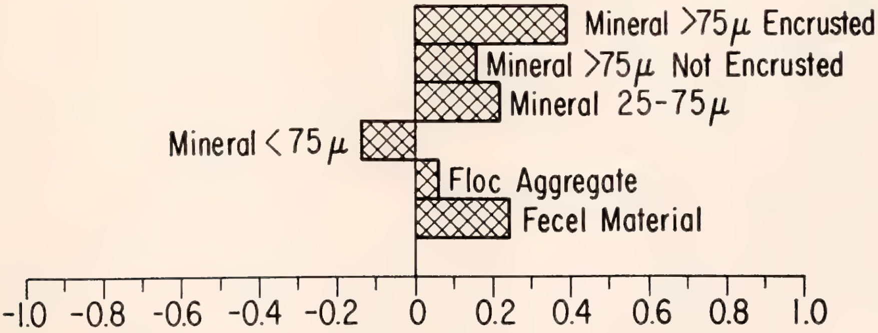

FIGURE 3. Electivity coefficients of different particle types selected by *Pectinaria gouldii* (data averaged for fifteen worms).

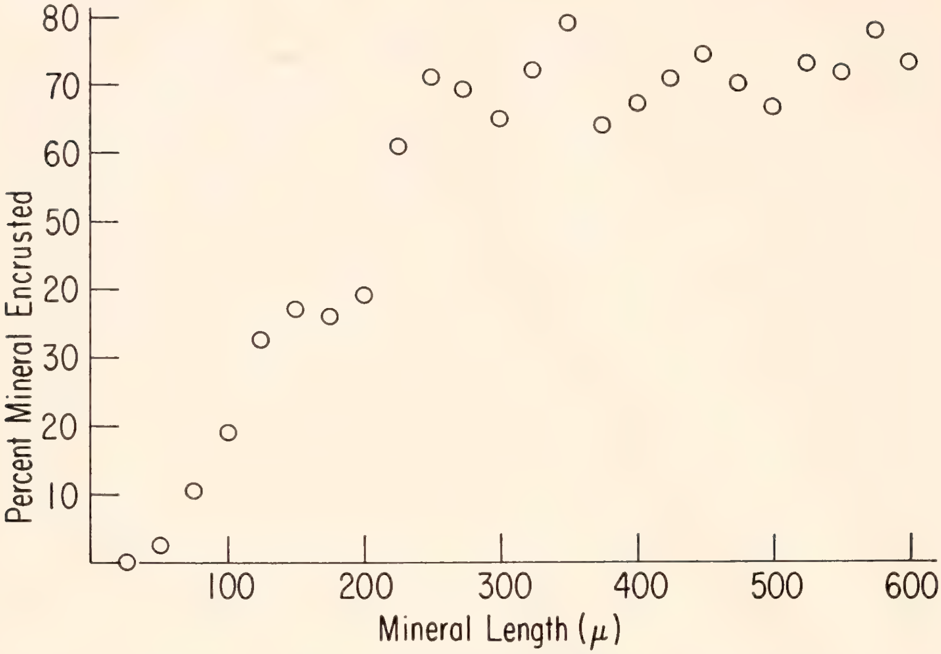

FIGURE 4. Relationship between mineral length found in the sediment where *Pectinaria gouldii* is feeding and percentages of minerals having encrustations. Mineral lengths are clumped into 25 μ size groups.

ingested). The data indicate that *P. gouldii* consistently prefers large encrusted mineral grains greater than 75 μ. Nonencrusted mineral grains greater than 75 μ and encrusted mineral 25–75 μ show positive electivity values in most of the worms. Fecal material and floc aggregates were selected in the majority of animals, though fecal material was selected more often. In all the polychaetes studied, there was avoidance (probably due to a mechanical or morphological constraint) for mineral grains less than 25 μ.

The percentage of encrusting material on the mineral grains is highly correlated with grain size (Fig. 4). Mineral grains less than 25 μ are never encrusted while grains greater than 75 μ are increasingly encrusted (10–80%). *P. gouldii* may be preferentially selecting large grains to increase the amount of organic matter ingested. As already noted, fecal material and floc aggregates are highly important food sources for deposit feeders and may be selected for the same reason.

Sediment staining

Table II gives the results of the periodic acid Schiff (PAS) staining of the sediment near the mouth, sediment ingested and sediment sampled from the intestine of *P. gouldii.* Analysis of the sediment samples near the mouth of the worms indicates an average of 32.7% of the particles are possible sources of organic matter. Possible sources of organic matter refers to the total material that could be regarded as a food source (as outlined above) for the polychaetes. The total

PAS stained material averages 13.9%. Of the 32.7% possible organic material, less than half (average of 42.6%) was stained by the carbohydrate stain suggesting that not all of the material is organic in nature. Studies by Waksman and Hotchkiss (1937), Anderson (1940), Mare (1942) and George (1964) have strongly suggested that only about 10–20% (by weight) of the organic matter in marine sediments is in a biologically utilizable form.

Analysis of the sediment from the foregut of the worms shows an average of 42.7% possible organic matter of which 20.5% was PAS stained. There was an average of 30.9% possible organic material obtained from the intestine of the worms of which 14.3% stained by the PAS method.

The mercuric bromphenol blue (MBB) method stained only a very small amount of material in the sediment. Of four samples analyzed, less than 0.4% of the material showed MBB reactions. Less than 0.1% of the ingested fraction obtained from the foregut of the worms stained with the MBB method, while sediment sampled from the intestine showed no reaction to this stain.

DISCUSSION

Calculations of the assimilation efficiency of *P. gouldii* show that the worms remove on the average 30% of the possible organic material from ingested sediment. Assimilation efficiencies of PAS-stained sediment average 29.1% for the fifteen worms. Gordon (1966) reports that *P. gouldii* removed almost 50% of the organic material (by weight) from each gram of sediment worked. George (1964) states that the deposit feeding polychaete *Cirriformia tentaculata* removes only 7.9% (by weight) of the organic matter present in the sediment. Adams and Angelovic (1970) found that the polychaete *Glycera dibranchiata* has an assimilation rate of 41% (based upon the amount of ^{14}C respired as CO_2) when fed detritus and 34% when fed undecomposed eel grass. Since the values presented in this study are not volumetric or by weight it is difficult to make comparisons with assimilation rates presented in the other studies. However, the values presented are well within the range of the other studies on assimilation efficiences.

TABLE II

Per cent particle abundance of carbohydrate staining (periodic acid Schiff)

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
Sediment near mouth of *P. gouldii*															
Per cent "possible" organic	38.9	37.3	31.1	33.1	32.4	31.2	25.9	30.8	32.1	36.5	33.3	29.1	36.5	29.4	33.0
Per cent stained	10.2	5.9	13.1	14.0	13.7	12.4	9.4	11.5	15.7	16.5	18.2	18.6	17.6	13.8	18.6
Per cent organic stained	26.2	15.8	42.1	42.3	42.3	39.7	36.3	37.3	48.9	45.2	54.6	63.9	48.2	46.9	56.4
Sediment in foregut of *P. gouldii*															
Per cent "possible" organic	41.9	37.9	48.1	45.7	36.5	43.3	53.9	41.6	46.1	40.1	47.3	42.2	43.5	36.2	36.9
Per cent stained	18.6	17.9	21.9	16.5	13.9	18.6	18.2	23.9	31.4	24.5	28.1	13.3	23.4	21.3	15.4
Per cent organic stained	44.4	47.2	45.5	36.1	38.1	42.9	33.8	57.5	61.2	61.1	59.4	31.5	53.8	58.8	41.7
Sediment in hindgut of *P. gouldii*															
Per cent "possible" organic	28.1	28.3	33.2	33.3	28.4	31.4	37.2	32.5	40.1	33.5	29.5	26.1	22.7	28.2	31.2
Per cent stained	12.9	13.1	16.1	11.7	11.4	8.9	17.0	17.2	27.0	17.9	13.5	11.7	15.3	10.0	11.4
Per cent organic stained	45.9	46.3	48.5	35.1	40.1	28.3	45.7	52.7	68.1	53.4	45.8	44.8	67.6	35.5	36.5
% "Possible" assimilated	33.2	25.3	30.9	27.3	22.2	27.5	30.9	21.9	13.0	16.4	37.6	38.1	47.8	22.1	15.4
% Stained assimilated	30.6	26.8	26.5	29.1	17.9	52.2	6.6	28.0	14.0	26.9	51.9	12.0	34.6	53.1	25.9

Data presented in this study provide some interesting results concerning the possible effects of the polychaete on the benthic environment. The worms concentrate an average of 32.7% possible organic material found in the sediment to 42.7% by selectively feeding. Through the concentration of organic material, the animals are channeling large amounts of organic material to the surface where it can become available to other organisms. The feeding activities of *P. gouldii* could have important effects upon the recycling of nutrients for the salt marsh tropic chain. The organic fraction deposited upon the surface may even be suspended in the water column. Rhoads (1973), for example, has shown that resuspended fecal material of benthic deposit feeders in Long Island Sound may represent a significant food source for commercially important suspension feeding molluscs.

The use of histological staining techniques for examination of sediments is a biologically meaningful way to ascertain the qualitative and quantitative composition of marine sediments. The staining methods, although very generalized in their staining affinities, are valuable in determining the nature of the food sources for benthic animals and those portions that may be biologically utilizable.

I wish to thank Drs. R. G. Johnson and T. J. M. Schopf for reviewing and improving the manuscript. This research was supported by NSF grant GA 35819 to Ralph G. Johnson, University of Chicago and the Marine Biological Laboratory.

SUMMARY

1. A study of the food-resource partitioning in the deposit-feeding polychaete *Pectinaria gouldii* collected from Little Sippewisset salt marsh, Massachusetts, shows that, on the average, larger worms select larger particles than smaller worms. Comparisons of ingested sediment with sediment collected where the animals were feeding indicate that the polychaetes prefer organic-encrusted mineral grains, floc aggregates, and fecal material.

2. Histological stains were used to determine the percentage particle abundance of different possible food sources and fractions ingested by the polychaetes. Mercuric bromphenol blue (MBB) was used to stain protein-containing material and periodic acid Schiff reagent (PAS) was used to stain carbohydrate-protein complexes. Total possible organic material in the sediment averaged 32.7%. Very little of the sediment (less than 0.4%) stained with MBB, while an average of 13.9% of the sediment stained with PAS. Of the total possible organic matter, only about one-half stained with the PAS reagent suggesting not all of the material is organic in nature.

3. Analysis of the sediment ingested by the worms averaged 42.7% possible organic matter, of which 20.5% was PAS-stained. Calculations of the assimilation efficiencies of *P. gouldii* show that the worms remove, on the average 30% of the possible organic matter and 29.1% of the stained material from the sediment.

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THE EFFECTS OF HEAVY METAL IONS ON THE MOTILITY OF SEA URCHIN SPERMATOZOA[1]

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Intense activity of sea urchin spermatozoa follows dilution in sea water. The high performance level is of limited duration; metabolism and motility decline to a low steady state and fertilizing capacity diminishes progressively with time (Tyler and Tyler, 1966). This so-called "dilution effect" has been attributed to the action of trace amounts of heavy metal ions present in seawater on the respiratory enzymes of the spermatozoa (Rothschild and Tuft, 1950; Rothschild and Tyler, 1954). Although there are numerous reports on the effect of various metal ions on the O_2 consumption of sea urchin spermatozoa, quantitative data on the action of heavy metals on spermatozoan motility are not generally available.

Rothschild and Tuft (1950) reported that small amounts (4×10^{-5} M) of $CuCl_2$ or $ZnCl_2$ added to dense suspensions ($> 4 \times 10^8$ sperm/ml) of *Echinus esculentus* spermatozoa increased their level of O_2 consumption, but had no effect on the respiration of dilute suspensions ($< 4 \times 10^8$ sperm/ml). While these authors did not provide actual measurements, they stated that neither copper nor zinc had any effect on the swimming speed of the spermatozoa.

Mohri (1956) found that $CuCl_2$ or $ZnCl_2$ at low concentration (10^{-5} M) accelerated the O_2 uptake of *Hemicentrotus pulcherrimus* spermatozoa, but at a higher concentration (10^{-4} M) inhibited it. According to Barron, Nelson, and Ardao (1948), 5×10^{-6} M $HgCl_2$ stimulates the respiration of *Arbacia punctulata* spermatozoa, but a concentration of 10^{-4} M inhibits their O_2 consumption. These investigators did not relate the effects on O_2 uptake to the motile activity of the cells.

Additional evidence for the sensitivity of sea urchin speramtozoa to the toxic effects of heavy metal ions comes from experiments on the ability of chelating agents to prolong both the motility and fertilizing capacity of sea urchin spermatozoa. Rothschild and Tyler (1954) found that 10 micromolar ethylenediaminetetraacetic acid (EDTA) depressed dilution-induced increase in O_2 uptake and delayed senescence of *Echinus esculentus* spermatozoa. Tyler (1953) used a variety of agents which bind heavy metal ions—amino acids, diethyldithiocarbamic acid (DEDTC), 8-hydroxyquinoline (oxine), and α-benzoinoxime (cupron)—to prolong the life span and fertilizing capacity of sea urchin and sand dollar spermatozoa. Mohri (1956) also found that chelating agents would suppress the respiratory increases observed on dilution of *Hemicentrotus pulcherrimus* sperm. The investigators concluded that these agents act to depress respiration, and thereby prolong sperm motility and viability, by binding heavy metals ordinarily present in the seawater.

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Although it is often assumed that increased O_2 consumption by the spermatozoon is accompanied by increased motile activity and decreased O_2 consumption by decreased motile activity both Robbie (1946) and Rothschild (1948) have demonstrated that spermatozoan motility is not a simple function of oxygen uptake.

In this report, using a convenient and objective determination of swimming speed, we explore quantitatively the effects of alterations in the environmental concentrations of heavy metal ions on the motile activity of *Arbacia punctulata* spermatozoa.

Materials and Methods

Spermatozoa were collected from sea urchins, *Arbacia punctulata*, by injecting 0.53 M KCl into the perivisceral cavity. The shed sperm were concentrated at 1000 × *g* in a clinical, table top centrifuge for 3 minutes, the supernatant fluid removed, and the packed sperm stored at 4° C. Immediately prior to the motility rating tests, samples of the spermatozoa were diluted to a final concentration of $10^7 \pm 10\%$ sperm/ml of filtered seawater, determined by optical density measurements of the sperm cell suspensions (Nelson, 1972). This concentration was selected empirically because it fell within the optical density range appropriate to the motility determinations.

Five milliliters of seawater suspensions of spermatozoa were added to round colorimeter tubes containing either: (1) 0.5 ml distilled water as control, (2) 0.5 ml 1% formaldehyde to kill the cells, or (3) 0.5 ml of the reagent being tested. The contents were mixed thoroughly by inverting twice.

The swimming speed of the spermatozoa was determined by the centrifuge-orientation, optical density method using a Bausch and Lomb Spectronic 20 Colorimeter at a wavelength of 540 nm and a clinical centrifuge. Measurements of sperm cell density were made immediately after mixing the sperm suspensions into the reagents and after each of three four-minute centrifugations at 120 × *g*. The method depends on the fact that, under the force generated in a mild gravitational field, the normally, slightly positively geotropic *Arbacia* sperm cells become oriented centrifugally and swim with decreased randomness to the bottom of the tube. While the dead cells also become similarly oriented, they no longer swim and are only minimally sedimented at 120 × *g*. Therefore, the rate of decrease of optical density (ΔOD), which was previously determined to be linearly proportional to the swimming speed, may be calculated after correction for any slight displacement of non-motile cells, and in comparison to the ΔOD of the untreated control cells, viz., $M = \Delta OD_x / \Delta OD_c \times 100$. Changes in the speed of the swimming sperm cells are expressed in terms of per cent of control swim rate.

All experimental procedures were carried out in an air-conditioned room (22–23° C) and initial swimming speeds were obtained within 5 minutes of dilution of the spermatozoa in filtered seawater. Branham (1966) and Timourian and Watchmaker (1970) have reported that the motile activity of sea urchin spermatozoa remains stable for approximately 30 minutes after dilution in seawater. The reagents tested included: $CuCl_2$ 500 nM to 100 μM, $ZnCl_2$ 5 μM to 10 mM, $MnCl_2$ 5 μM to 10 mM, $HgCl_2$ 10 nM to 1 mM, EDTA 10 μM to 5 mM (adjusted to pH 7.8 with NaOH before use). The natural concentrations, in μEq/liter, of

TABLE I

Dose- and time-dependence of zinc effects. Motility, expressed as per cent of control motility (100%), of Arbacia punctulata *spermatozoa after each of a series of three 4-minute centrifugations at 120 × g; 10^7 ± 10% sperm in 5 ml filtered Woods Hole seawater; temperature 22–23° C. Each value represents the mean of individual determinations made on spermatozoa collected from three different* Arbacia punctulata, ± *standard deviation*

Zinc added in μEq/liter	% Control motility		
	Centrifugation #1	Centrifugation #2	Centrifugation #3
2500	22 ± 5.6	20 ± 5.6	20 ± 4.8
500	36 ± 2.2	34 ± 2.8	30 ± 2.8
250	44 ± 2.6	29 ± 0.6	17 ± 4.1
50	80 ± 7.2	52 ± 5.8	30 ± 4.6
25	155 ± 10.8	111 ± 4.6	97 ± 8.7
5	113 ± 5.0	105 ± 7.1	107 ± 10.6
2.5	98 ± 3.6	101 ± 1.0	100 ± 1.0

certain heavy metal ions in seawater (using data adapted from Richards, 1972) are zinc—0.3, copper—0.1, manganese—0.1, and mercury—0.002.

RESULTS

Zinc

Figure 1 shows the response of *Arbacia punctulata* spermatozoa to increases in environmental zinc. Sea water contains approximately 0.3 μEq zinc/liter (Richards, 1972); increasing the zinc concentration to 5 μEq/liter causes little more than a 10% increase in swim speed while a tenfold increase in zinc concentration (to 25 μEq/liter) speeds up the sperm by 55%. Increasing the amount of environmental zinc to 50 μEq/liter slows the swimming speed of the spermatozoa to 20% below control values. As the concentration of zinc is increased from 250 to 500 to 2500 μEq/liter of seawater, motility declines from 44% to 36% to 22% of control levels. However, we had to increase the concentration of zinc in the medium to 15 mEq/liter in order to immoblize the sperm cells completely.

Prolonged contact with zinc ion, even at concentrations which initially stimulate motility, has an apparently debilitating effect on the motile activity of the spermatozoa. Table I shows that, following the addition of 25 μEq of zinc, *Arbacia* sperm cells swim at 155% of the control speed after the first four minutes centrifugation, 111% of the control swim rate after the second four-minute centrifugation. At 250 μEq zinc/liter, motility declines from 44% to 29% to 17% of the control speed. In both higher (0.5 to 2.5 mEq) and lower (2.5 to 5 μEq) concentrations of added zinc, there is no significant change in the swimming speed of the *Arbacia* spermatozoa after the first 4 minutes centrifugation.

Copper

The concentration of copper in seawater is equivalent to about 0.1 μEq/liter. The effect of increases in environmental copper on the swimming speed of spermatozoa collected from each of two sea urchins is illustrated in Figure 2. Addition of 0.5 μEq copper speeds the sperm by approximately 10%. In the presence of 2.5 μEq added copper, sperm motility falls to 86 or 66% of control values, decreases to 37 or 58% of control values in 5.0 μEq excess copper, to 31 or 36% of control values in 25 μEq excess copper, and ceases completely in the presence of 50 μEq

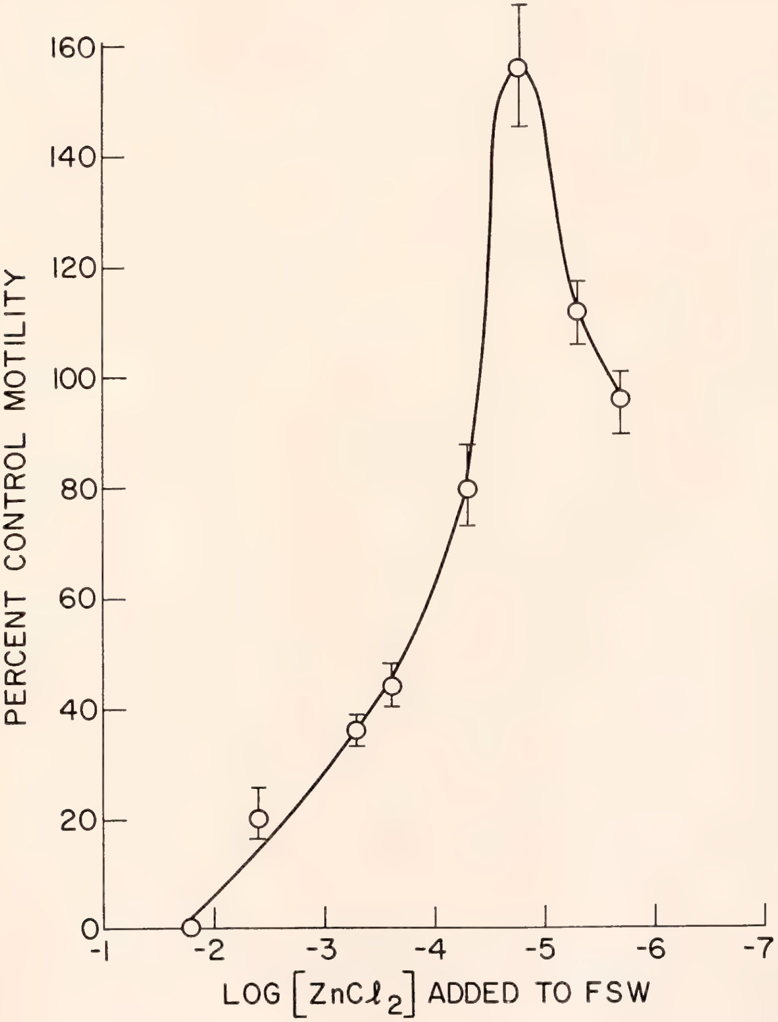

FIGURE 1. Effect of $ZnCl_2$ on the swimming speed of *Arbacia punctulata* spermatozoa. Varying concentrations of $ZnCl_2$ added to filtered Woods Hole seawater containing $10^7 \pm 10\%$ *Arbacia punctulata* spermatozoa elicit a biphasic dose-dependent response. *Abscissa* represents the zinc chloride concentrations added, in log moles per liter seawater/sperm suspensions; *ordinate* is the swim rate of treated sperm expressed as per cent of the control rate in seawater. The points and ranges indicate the mean and standard deviations of individual determinations made on spermatozoa collected from three individual *Arbacia*. The incubation varied from 22–23° C on different days.

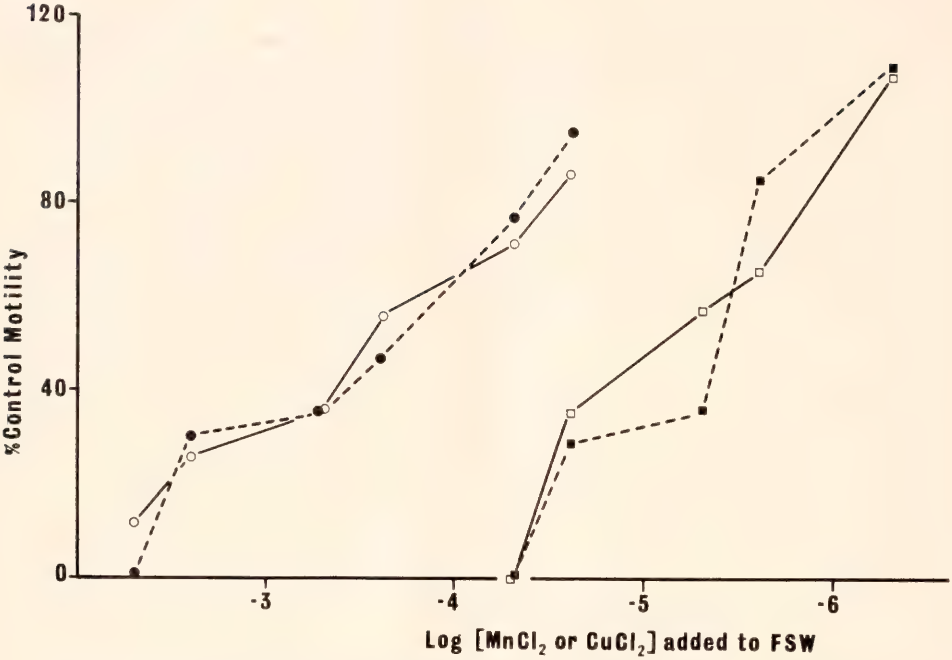

FIGURE 2. Effects of $CuCl_2$ and $MnCl_2$ on the swimming speed of *Arbacia punctulata* spermatozoa. $MnCl_2$ and $CuCl_2$ added to filtered Woods Hole seawater containing $10^7 \pm 10\%$ *Arbacia punctulata* spermatozoa decreased their swimming speed in proportion to the concentration. *Abscissa* is concentrations of metal ion added, in log moles per liter of seawater/sperm suspensions: copper—open and closed squares, manganese—open and closed circles (open and closed data points from replicate runs). *Ordinate* is the swim rate of treated sperm expressed as per cent of control rate in a seawater. Each point represents an individual determination of swim rate expressed as a per cent of control rate in seawater at a temperature of 22–23° C. Note that while the seawater content of Mn^{2+} and Cu^{2+} both equal about 10^{-7} equivalents per liter, the sperm cells are more sensitive to excess copper by about two orders of magnitude.

excess copper. Copper appears to exert its effect on sperm motility almost immediately after addition to the sperm cell suspensions; further significant declines in swimming speed do not occur after the first 4 minutes centrifugation.

Manganese

Woods Hole seawater contains approximately the same concentration of manganese as it does copper. But as shown in Figure 2, manganese exerts much less of an inhibitory effect on *Arbacia* sperm motility than does copper. Addition of 0.5, 2.5 or 5 μEq manganese/liter to the sperm cell suspensions does not measurably affect the swimming speed. Excess manganese up to 25 μEq/liter causes only a 4 or 15% reduction in motility and further increases result in only a slow decline in Arbacia sperm swimming activity; in the presence of 2.5 mEq manganese/liter—approximately three orders of magnitude greater than the normal seawater concentrations of this heavy metal ion—motile activity is still about one-quarter of the control value.

Mercury

The effect of $HgCl_2$ on the motility of *Arbacia punctulata* spermatozoa was measured after incubation of the cells with mercuric ion for fixed intervals of time. Figure 3 (solid line) is an example of the changes in motility of sperm cells collected from a single sea urchin after 8 minutes incubation in 5×10^{-8} to 10^{-3} M/1 $HgCl_2$. A marked increase in swim speed is observed at mercury concentrations between 10^{-4} and 10^{-7} M. In 5×10^{-6} M $HgCl_2$ *Arbacia* sperm swim at about 270% of the control rate. In higher concentrations of mercury, motility declines but is still 10% above control values in 10^{-3} M $HgCl_2$. As the concentration of mercuric ion is decreased below 10^{-7} M, acceleration gradually declines and the

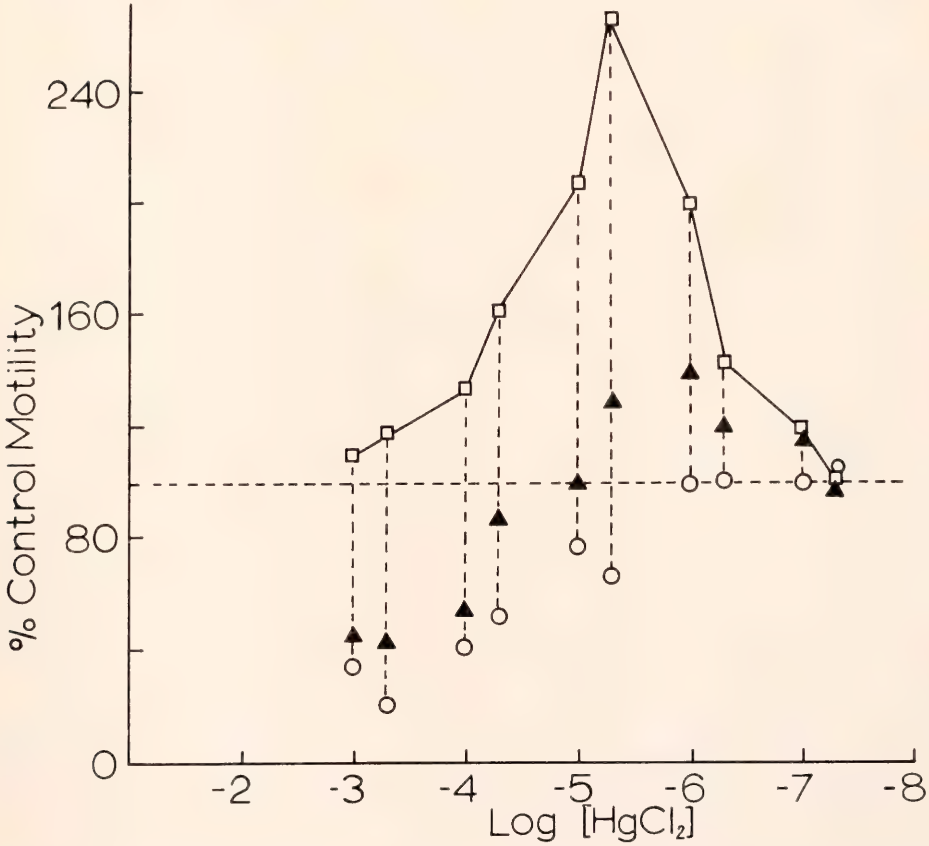

FIGURE 3. Effect of $HgCl_2$ on the swimming speed of *Arbacia punctulata* spermatozoa. The $HgCl_2$ added to filtered Woods Hole seawater containing 10^7 + 10% *Arbacia punctulata* spermatozoa shows pronounced dose-dependent and time-dependent effect. The *abscissa* is the log molar concentration of mercury in seawater/sperm suspensions: open square—8 minutes incubation; solid triangles—16 minutes incubation; open circles—24 minutes incubation. The *ordinate* shows the swim rate of treated sperm expressed as per cent of control rate in seawater, temperature 22–23° C.

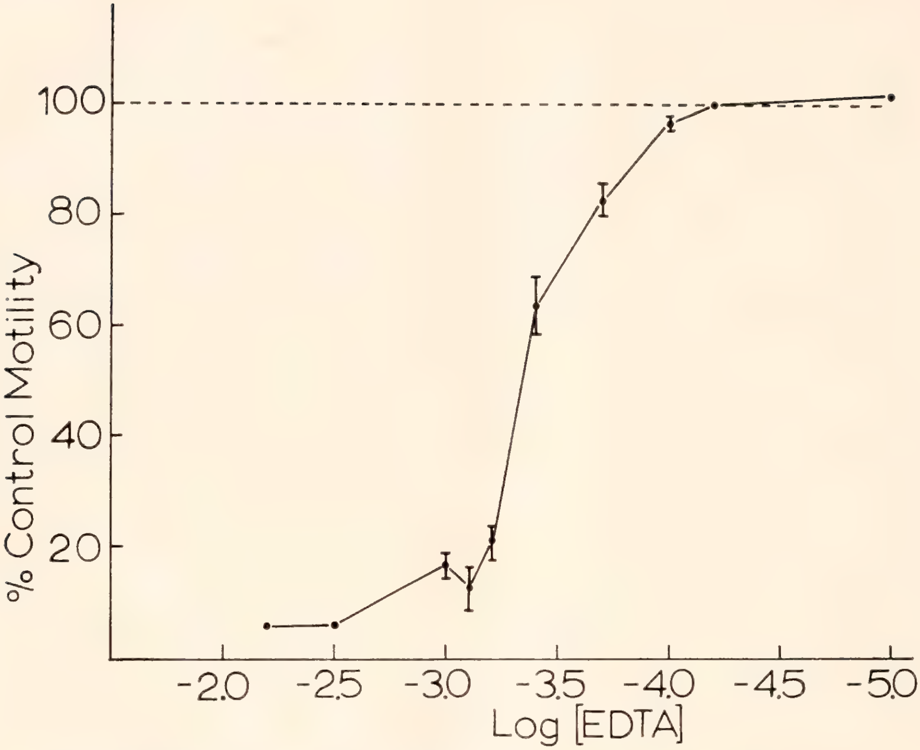

FIGURE 4. Effect of EDTA on the swimming speed of *Arbacia punctulata* spermatozoa. EDTA added to filtered Woods Hole seawater containing $10^7 + 10\%$ *Arbacia punctulata* spermatozoa per milliliter depresses the swimming speed at effective concentrations. The *abscissa* is in log molar concentration of EDTA in seawater/sperm suspensions. The *ordinate* is the swim rate of treated sperm expressed as per cent of the control rate in seawater. The points and ranges represent the mean and standard deviations of separate determinations made on spermatozoa collected from three individual *Arbacia punctulata*. Incubation temperature was 22–23° C.

effect is negligible at 5×10^{-8} M. Moreover, the stimulatory effect of $HgCl_2$ on *Arbacia* sperm motility is transitory. The dashed lines in Figure 3 show the decline in swim speed observed after 16 and 24 minutes incubation in various concentrations of $HgCl_2$. For example, in 5×10^{-6} M $HgCl_2$ motility is 170% above control level after the first centrifugation (8-minute measurement), 30% above control level after the second centrifugation (16-minute measurement) and 33% below control level after the third centrifugation (24 minutes measurement).

Since an initial stimulation and subsequent decline in sperm motility was observed at all concentrations of $HgCl_2$ tested, the effect of preincubation with mercuric ion on sperm swimming speed was determined. The results of a ten-minute preincubation of the sperm cells in 10^{-3} to 10^{-7} M $HgCl_2$ essentially confirm the observations recorded on sperm cell delayed response illustrated in Figure 3.

EDTA

EDTA, in excess of 100 μM/l of seawater, exerts a profoundly depressant effect on the swimming speed of sea urchin spermatozoa (Fig. 4). Motility was measured in concentrations of EDTA ranging from 5×10^{-3} to 5×10^{-5} molar. In Figure 4 we note the following: in seawater suspensions containing less than 10^{-4} Moles of EDTA per liter, motility appears normal. A twofold increase in EDTA concentration, to 2×10^{-4} M, causes a 17% decrease in the swimming speed of the spermatozoa. Another doubling of the EDTA concentration, results in a further decline in motility to 64% of control values. In 6×10^{-4} M EDTA swim speed declines to only 21% of control rates. As the concentration of EDTA is further increased, the motility of the *Arbacia* spermatozoa continues to decline at a slow rate and in 2×10^{-3} M EDTA the sperm are virtually immotile.

Discussion

From the evidence we may conclude that the motile activity of *Arbacia punctulata* spermatozoa is affected in a concentration-dependent and, in some instances, time-dependent, manner by the heavy metal ion composition of the environment.

Although we are not prepared at present to specify the particular sites within the spermatozooon on which these metal ions exert their action, previous studies indicate that the effects on motility may in part depend on heavy meal interaction with protein sulfhydryl groups to form mercaptides. Barron *et al.* (1958) suggested that soluble —SH groups are necessary for the activity of enzymes essential for spermatozoan viability.

Sea urchin spermatozoa exhibit a biphasic response to increases in the environmental concentration of zinc ions. A three- to thirtyfold increase of the zinc in the seawater causes the *Arbacia punctulata* spermatozoa to swim between 13 and 55% faster than control sperm in normal seawater. This acceleration of the swim rate is only transitory since, within 30 minutes of the addition of 5 to 25 μEq zinc/liter to the seawater in sperm suspensions, motility has declined to control levels. Larger excesses of zinc, above 50 μEq/liter, do not stimulate sperm motility, but instead cause an immediate fall in swimming speed which continues to decline as the time of incubation in zinc ion is increased. Zinc, at a concentration of 25 μEq/liter, which according to Rothschild and Tuft (1950) has no effect on the oxygen uptake of dilute suspensions of *Echinus* spermatozoa, exerts a short-term stimulatory effect on the motility of *Arbacia* spermatozoa. Therefore zinc is probably acting in other ways than only by combining with the soluble sulfhydryl groups involved in regulation of sperm cell metabolism. Morisawa and Mohri (1972) found that both the sperm tails and isolated microtubules of the sea urchin *Pseudocentrotus depressus* appear to concentrate zinc, and they concluded that this ion may play an important role in the contractile process. The fairly large excess of zinc needed to overcome the initial acceleration of *Arbacia* sperm motility indicates that, at concentrations above 50 μEq/liter, zinc's inhibitory effect may be due to binding at active sites on contractile proteins. In fact, Utida and coworkers (1956) report that other divalent cations, Cd^{2+}, Co^{2+} and Ni^{2+}, increase ATP hydrolysis by washed sperm tails of the sea urchin *Heliocidaris crassispina* at 1 mM/l, while Cu^{2+} and Zn^{2+} activate the enzyme at 10^{-4} molar but inhibit at the higher concentration. Our data on swimming speed (see Fig. 1) bear a most striking resemblance to the graphic representation of Utida, Maruyama and Nanao (1956) relating sperm tail apyrase activity to the zinc concentration.

In the range that we examined, both copper and manganese ions inhibit *Arbacia* sperm motility, but while we found copper to be about 100 times more potent an inhibitor of sea urchin sperm swimming than was managanese, still, at the lowest concentration—0.5 μM—$CuCl_2$ actually stimulates motility by about 10%. An average hundredfold increase above environmental manganese causes an 8% decrease in swim speed while an equivalent increase in the copper content of seawater depresses *Arbacia* sperm motility by 77%. The comparatively large amounts of manganese which must be added to the sperm cell suspensions before significant changes in motile activity can be detected suggest that manganese may, among other things, have only limited access to active groups which are important for propulsive activity. Alternatively, we cannot overlook the report of Garbers, Lust, First and Lardy (1971) that the sperm cells of the sea urchin, *Stronglyocentrotus* are remarkably endowed with guanyl cyclase, and to a lesser extent, adenyl cyclase, both of which are highly Mn^{2+} dependent. Agents which influence cyclic nucleotide metabolism affect both motility and respiration of spermatozoa according to Garbers *et al.* (1971).

Since small amounts of copper (2.5 μEq/liter) in the seawater/sperm suspensions cause a measurable decrease (34%) in swim speed, it seems likely that copper may act at essential regulatory sites within the spermatozoan flagellum. The role of copper in metabolism is well documented; excess copper ions exert their effects at a variety of enzymatic sites. Morisawa and Mohri (1972) believe that cytochrome C oxidase in the midpiece of sea urchin spermatozoa accounts for most of the copper found in these sperm while Barnes and Rothschild (1950) claim that sea urchin sperm are able to bind copper to the extent of 300 times the amount in seawater. Rothschild and Tuft (1950) have shown that copper arrests the decline in oxygen consumption of dilute suspensions of sea urchin spermatozoa.

Mercury, in contrast to zinc and to managanese and copper, had not until recently been regarded as a biologically significant constituent of seawater. Nevertheless this heavy metal ion is becoming increasingly important as a pollutant. We found *Arbacia* sperm cells to be extremely sensitive in both dose-dependent and time-dependent manner to supplements of mercury. Addition of mercuric chloride to 5×10^{-6} M (or 3×10^{8} molecules of mercury salt/spermatozoon at a working dilution of 10^{7} sperm/ml seawater), accelerates the sperm by about 170%. This increase, however, is only transitory and the mercuric ion evinces its ultimate toxicity by causing the abrupt fall in motile activity that rapidly supersedes the initial rise. For example, prolonging the incubation period from 8 to 24 minutes in 5×10^{-6} M mercury causes about a 200% decrease in the swimming speed of the sea urchin sperm from 170% above control values after 8 minutes incubation, to 29% above control values after 16 minutes incubation, to 33% below control values after 24 minutes incubation in mercury. Barron *et al.* (1948) observed that low concentrations of mercury (5×10^{-6} M) caused an 88% increase in the respiratory activity of *Arbacia punctulata* spermatozoa while higher concentrations of mercury (10^{-4} M) caused complete inhibition of the respiration. This they attributed to the fact that low levels of mercury, in combining with soluble $-SH$

groups that are important in the regulation of cell respiration, abolish that regulatory function, while with further increases, the mercury next combines with fixed $-SH$ groups on essential proteins to inhibit enzymatic activity. Although we observed that low concentrations of mercury (5×10^{-6} M) caused an initial large increase in motile activity, we were still able to record motile activity in spermatozoa suspended in seawater containing mercury concentrations as high as 10^{-4} M. When exposed to this concentration of mercuric ion, the sperm swam at 133% of control rate after 8 minutes, at 54% of control after 16 minutes, and at 41% of control after 24 minutes exposure.

Nevertheless some critical amount of certain of the heavy metal ions must be necessary for optimum motility since EDTA drastically reduces the swimming speed of dilute suspensions of *Arbacia* spermatozoa. The effect of EDTA in increasing the life span and fertilizing capacity of sea urchin spermatozoa by removing trace metals from seawater has been discussed by several investigators (Rothschild and Tyler, 1954; Mohri, 1956; Tyler, 1953). Rothschild and Tyler (1954) calculate that one micromole/liter of EDTA is sufficient to bind all the trace metal ions in ordinary seawater. In our experiments, 10^{-6} M EDTA had no effect on the swimming speed of *Arbacia* spermatozoa. However, the spermatozoa used in this study were in dilute suspension and it is probable that low steady state conditions already prevailed even though we completed our first measurements within 5 minutes of dilution. Decreases in swimming speed did not become apparent until the EDTA was raised to 2×10^{-4} M/l. At this concentration motility was 83% of control values while in 8×10^{-4} M EDTA, motility further decreased to 13% of control values. Since EDTA did not affect *Arbacia* sperm swim speed except at high concentrations and since the concentration range of its effectiveness was sharply limited, it is possible that EDTA brought about a decrease in sperm motility by interfering with transport of divalent cations from cell surface sites as well as by removing trace metals from the seawater.

In this paper we have shown that, although a critical level of certain heavy metal ions must be maintained for optimum sea urchin sperm motility, an excess of these ions in the seawater adversely affects the propulsive activity of the spermatozoa. While metal ions can induce changes in motility, the mechanism of their effects on specific sperm enzymes and on the fertilizing capacity of the sea urchin spermatozoa remains to be investigated. In any case, as a probable consequence of the accumulation of heavy metals as pollutants in our oceans, large deficits in the reproductive capabilities of marine invertebrates will begin to become more apparent.

SUMMARY

Optimum motility of sea urchin spermatozoa for a period adequate to initiate the process of fertilization requires an apparently critical level of certain heavy metal ions. Increase of some of the divalent cations above the "normal" seawater content accelerates or depresses the swimming speed in dose- or time-dependent fashion (or both). The different patterns of *Arbacia* sperm swimming speed response to the individual cation supplements (Cu, Zn, Mn, Hg) may reflect differences in rate of penetration into the cell, binding of atcive groups or selective inhibition of as yet unspecified enzymes at or below the cell surface which directly

or indirectly contribute to regulation of flagellar contractility. The concentrations tested ranged from 500 nM up to 10 mM. The "optimum" concentrations fell between 1 to 10 micromolar Cu, Zn and Hg on initial exposure, while Mn was moderately inhibitory at these levels. EDTA, up to 10^{-4} molar, exerts no adverse effect on sperm propulsion, while 8×10^{-4} M almost completely blocks the motility. Within this short concentration span, the EDTA appears not only to bind essential seawater cations, but may also deplete those intracellular regulatory cations which otherwise may exist in a state of dynamic equilibrium with the seawater.

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THE PROCESS OF EGG-LAYING IN THE CHAETOGNATH *SAGITTA HISPIDA* [1]

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The process of egg-laying was described in *Sagitta bipunctata* by Boveri (1890), Stevens (1905, 1910), Bordàs (1920) and Ghirardelli (1968), in *S. hispida* by Conant (1896), in *S. hexaptera* by Grassi (1883), in *S. elegans* by Stevens (1905, 1910) in *S. setosa* by Dallot (1967, 1968), in *S. inflata* by Ghirardelli (1968), and in *Spadella cephaloptera* by Vasiljev (1925) and Ghirardelli (1968).

These authors do not agree as to how the ovum is fertilized by the sperm, from which it is separated by a layer of germinal epithelium known as the crescent. Disagreement also exists concerning the manner in which the fertilized egg reaches the exterior, the nature of the opening through which it leaves the body, and even on the number of ducts in the female reproductive system.

Reeve and Walter (1972a) described mating in *Sagitta hispida*, during which one or both spermatophores may be exchanged, following which the sperm migrate down the body of the animal to the nearest gonopore. Upon reaching it, they separate into two groups, one of which enters the adjacent gonopore while the other crosses over the body to the gonopore on the opposite side. The sperm enter the gonopores and move rapidly up a duct which lies on the outer side of each ovary.

The observations reported below describe events beyond this point which result in the laying of eggs, and indicate that in *Sagitta hispida*, at least, there is one tube which serves both as a sperm duct and an oviduct, and that the eggs are extruded by contractions of the ovary wall and body wall.

METHODS

Sagitta hispida Conant, a neritic species of chaetognath of approximately 10 mm mature length, is readily obtainable in plankton tows made from the laboratory dock on Biscayne Bay (Miami), and can be cultured in the laboratory for periods of several weeks. The detailed methodology of culture was provided by Reeve and Walter (1972b). In summary, animals were maintained in transparent acrylic 80-liter aquaria and fed natural zooplankton. The observations reported below were made on many such populations over a period of two years by withdrawing organisms from aquaria, placing them in small, shallow dishes or upon slides, and observing them live under the microscope. Of hundreds of animals examined in this way, numerous photomicrographs were made of organisms at various stages in the sexual cycle. In some cases individual animals were retained for several hours up to 2 days in 250-ml containers for further observation on the progress of egg-laying in the live animal. At least 30 animals, having been examined at various stages of egg-laying, were fixed in Bouin's solution, sectioned (8 μ) and stained with eosin and haematoxylin. This material was examined and photographed up to magnifications of × 1000. The external appearance of the gonopore region was also examined by scanning electron microscopy after preparation as described by Cosper and Reeve (1970). Observations on egg deposition were made on mature populations of chaetognaths in 80-liter aquaria into which had been transplanted three of the commonest local benthic plants (*Thalassia testudinum*, *Syringodium filiforme* and *Diplanthera wrightii*). The surfaces of the aquaria and of the plants (the latter having been removed to dishes) were examined for the presence of eggs after 48 hours.

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OBSERVATIONS

Fertilization

Following mating, sperm can be seen in live animals examined microscopically (see figures in Reeve and Walter, 1972a) making vigorous movements in the duct running along the outer side of each ovary, and in prepared sections (Figs. 1A and B). Although the act of fertilization was not seen, at the cellular level, it appeared to be effected by passage of the sperm through a stalk consisting of two cells (Fig. 1A) which appear, in section, to be highly vacuolar, and somewhat indistinct at their boundaries, as though on the point of degenerating. The stalk, (accessory fertilization cells, see Discussion) is part of the germinal epithelium from which the immature ovum grows out, and through which it maintains an attachment to the epithelium as it develops.

Migration of mature eggs out of the ovary

The egg in Figure 1B is fully mature and about to migrate from ovary to duct; it is separated from the duct by only a thin strand of germinal epithelium.

In order to reach the duct, the egg must pass through the germinal epithelium. It appears to do this following degeneration of the stalk cells, or their being engulfed by the egg, by insinuating itself very gradually (Figs. 1C–F) through the resulting pore. First the egg becomes oval, rather than spherical, and then it becomes pear shaped. The small lobe of the pear is pushed through the pore and into the duct. Once through, it is gradually enlarged until the major part of the egg has migrated, the egg being somewhat dumb-bell shaped during this process. Then the remainder of the egg slips through, till the migration is complete.

This process seems to us to be accomplished by several means. The egg itself appears to make squeezing movements, though these are perhaps of too precise a nature to be termed ameboid. Also we have observed peristaltic waves of contractions passing along the ovary from anterior to posterior together with the appearance of bands circumscribing the ovary wall (Fig. 1G). Possibly these contractions help to propel the eggs through the crescent. In addition, pressure of the gut and even the normal muscular contractions of swimming movements may aid the eggs in their migration. The time required for all the mature eggs to complete their migrations, which are performed randomly rather than in unison, varies from 15 to 20 minutes.

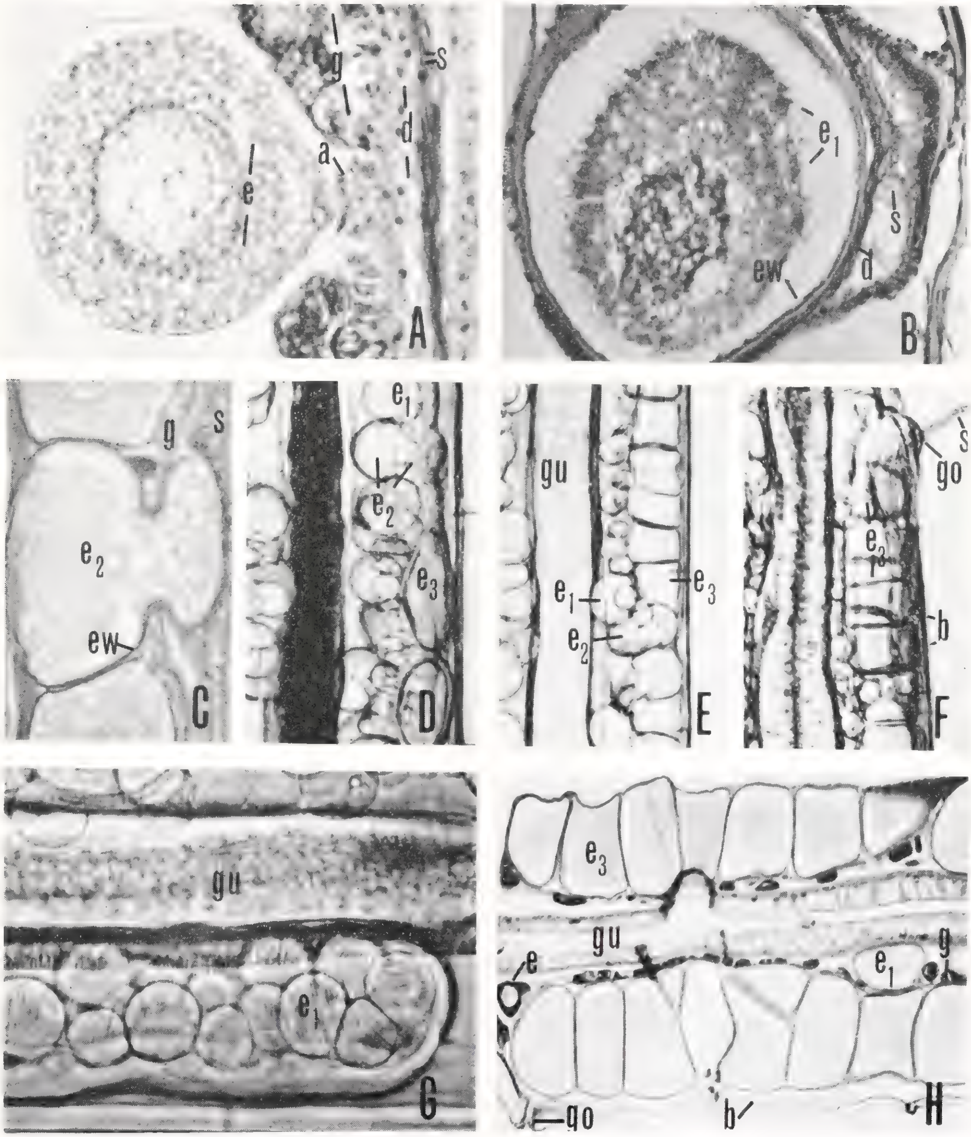
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FIGURE 1. A—maturing egg; B—mature egg; C to F—stages in migration of egg to duct; G—ovary with mature unmigrated eggs showing contractions of ovary walls; H—eggs in duct immediately prior to laying. Figures A–C and H are from sections, D–G from live material; width of body approximately 0.5 mm; a—accessory fertilization cells; b—body wall; d—duct; e—maturing egg; e_1—mature unmigrated egg; e_2—migrating egg; e_3—migrated egg; cw—egg cell wall; g—germinal epithelium; go—gonopore; gu—gut; s—sperm.

As more and more eggs pass through the germinal epithelium into the duct, they become crowded (Figs. 1D, E and F), possibly because the lumen of the duct is narrower than the diameter of the eggs. They are compressed, as Conant (1896; page 82) described it, "from before backward" until their shape is cylindrical rather than spherical. When all the eggs have migrated, the duct is completely filled with eggs (Fig. 1H) so that, viewed from above, it has the appearance of a ladder, the boundaries between two adjacent eggs froming the rungs, and the walls of the duct and the outer boundaries of the eggs forming the uprights of the ladder. The immature eggs in the ovary are compressed against the gut (Figs. 1H and 2B). The animal will now lay its eggs within 10 or 15 minutes. In sections of individuals fixed at this stage there is no evidence of any duct other than the one occupied by the eggs (Figs. 1H and 2B).

Animals which are almost ready to lay eggs often attach to the side walls of the aquarium but they do not necessarily remain continuously attached till the laying is accomplished, but rather shift position every few minutes. Even animals whose eggs are migrating tend to move sporadically about, and we suggest that the muscular contractions of the body wall may aid in compressing the ovaries so that the eggs may more easily migrate from ovary to duct.

The duct

In sections of inseminated animals whose eggs have already migrated through the germinal epithelium, sperm may be seen directly adjacent to the cell wall of the egg (Figs. 2B and C). In addition to the direct visual evidence that sperm are not separated from eggs when the latter emerge on the outer side of the crescent, at 1000 magnifications, even under low power sperm bulges can be seen where they are trapped between two adjacent eggs or forced anteriorly into the blind end of the duct. Indeed, it is not unusual to see bundles of motile sperm extruded to the exterior (Fig. 1F) as eggs migrate through the crescent. There is, therefore, no evidence that eggs and sperm occupy different ducts.

The gonopore

Observations of the gonopore, both in sections (Fig. 2B) and with the scanning electron microscope, indicate that it has only one opening, and that only one duct connects with it. Sections of animals in which eggs have migrated prior to being laid (Figs. 1H, 2A and B) clearly show the terminal egg in the cavity of a duct continuous with the opening of the gonopore, and where sperm are present (Fig. 2B) show them to occupy the same cavity.

Expulsion of mature eggs

Our observations indicate that the eggs are expelled by a sudden and intense contraction of the ovary and body walls. We are indebted to our colleague, M. A. Walter, for a photograph of a live animal (Fig. 2D) in the process of expelling its eggs, in which the body wall contractions are clearly visible. Contractions of the ventral body-wall musculature in the region of the gonopores (Fig. 2A) is presumably also involved, by expanding the orifice of the gonopores so that the eggs

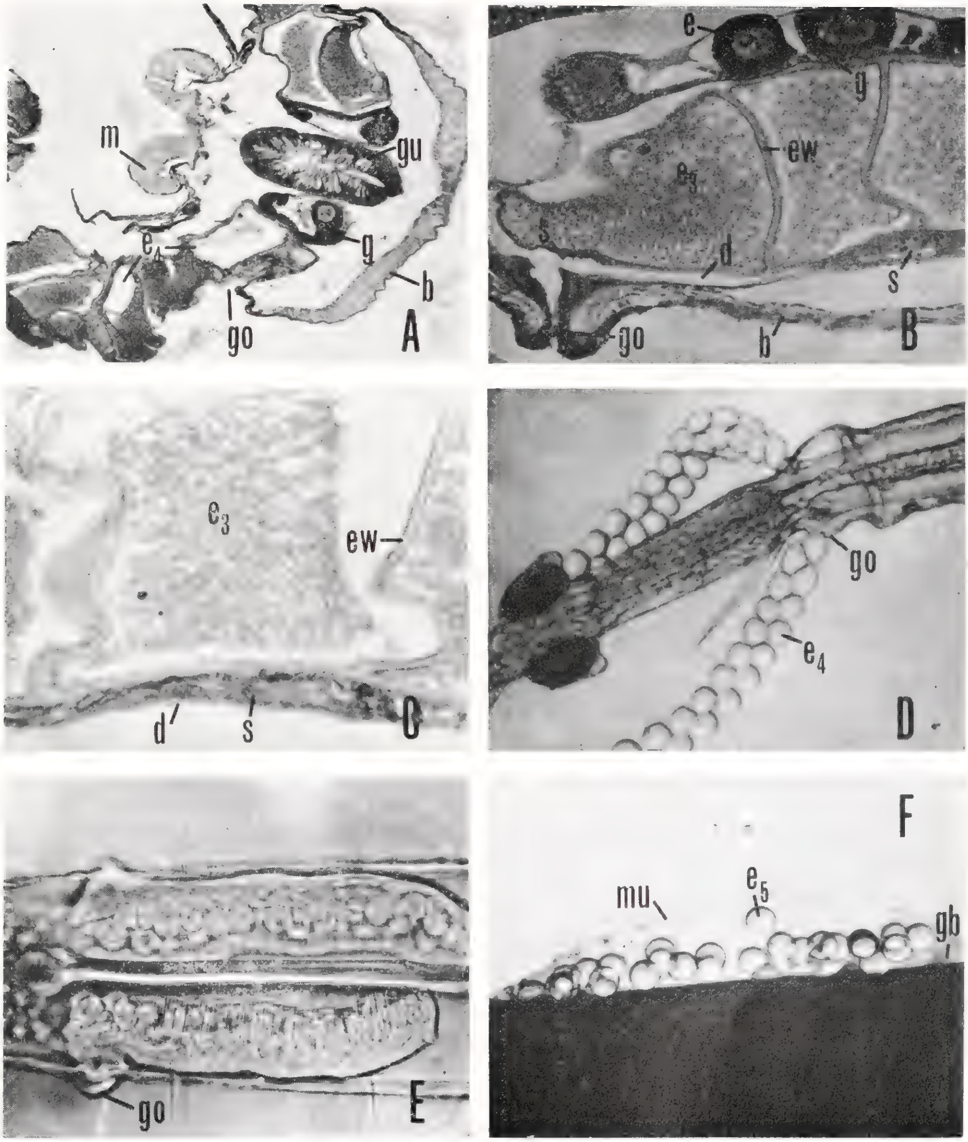
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FIGURE 2. A—eggs in process of being laid from gonopore; B—eggs about to be laid adjacent to gonopore; C—sperm and eggs in duct; D—eggs being laid; E—ovary showing contraction bands after egg-laying; F—mucus-embedded eggs attached to grass blade. Figures A–C are from sections, D–F from live material. Diameter of eggs approx. 0.2 mm. e_4—eggs in process of being laid; e_5—attached (laid) eggs; gb—grass blade; m—muscle; mu—mucus, Other abbreviations as in Figure 1.

do not have to deform their shape (Fig. 2A) as much as they did in order to pass through the crescent.

The eggs issue in two streams from each gonopore (Fig. 2D), and not, as Conant (1896) reported, one from each gonopore, so that the egg mass ultimately has the appearance of four strands of beads. The eggs are laid about ten or fifteen minutes after the last one has migrated through the germinal epithelium (at 26° C).

Following laying, the ovaries appear crumpled and contracted in an anterior-posterior plane; they are wide, filling the diameter of the body cavity but not even extending as far forward as the anterior of the posterior fin (Fig. 2E). They may be distinguished from those of a less mature animal which has yet to lay eggs (see Reeve and Cosper, 1974), for in the latter the ovaries are narrow and smooth-walled. As noted elsewhere (Reeve and Cosper, 1974) *Sagitta hispida* does not shed its eggs into the water column. In the laboratory strings or clumps of eggs attach as they emerge to the side walls of the aquarium, or to the bottom in the case of small dishes. When three common representatives of the local benthic macroflora (*Thalassia testudinum*, *Syringodium filiforme* and *Diplanthera wrightii*) were placed in aquaria with mature chaetognaths, we observed that the animals would attach to and deposit their eggs on the vegetation (Fig. 2F) provided that the latter was securely anchored to the bottom of the container.

DISCUSSION

In order to account for the method by which the sperm reaches to ovum, Buchner (1910) and Stevens (1903, 1905) proposed the existence of two accessory fertilization cells, the outermost being inserted in the wall of the "oviduct" and the inner being inserted in the ovum. The sperm swims through a series of vacuoles in these cells from the "oviduct" to the ovum. Ghiradelli (1968) confirmed these observations in *Sagitta bipunctata* and *Spadella cephaloptera*.

Our observations confirm those of Conant (1896) who suggested that the developing oocytes are attached to the ovary by a stalk (Fig. 1A) and contradict those of Stevens (1903) who maintained that the eggs develop free inside the ovary and then "each one becomes connected with two of the epithelial cells which are just lateral to the region" (page 232). This stalk later serves as a passage for the sperm to the egg as suggested by Stevens (1903), Buchner (1910), Vasiljev (1925) and Ghirardelli (1968).

Following fertilization, and indeed, even in its absence (Reeve and Cosper, 1974) the mature eggs move from the ovary into the duct which leads to the gonopore. Since they make this migration irrespective of whether copulation and fertilization has occurred, this process must be triggered by the state of maturation of the egg itself and not by its penetration by the sperm.

In *Sagitta* the egg must pass through the germinal epithelium of the crescent into the duct by which it gains access to the exterior. Exactly how the egg moves, or is moved, is uncertain, although Dallot (1968) has reported seeing contractions of the ovary wall, confirming Conant's (1896) earlier observations. Ghirardelli (1968) and Stevens (1905, 1910), however, maintained that the egg makes its way into the duct by ameboid movements. In *Sagitta hispida* the movement from ovary to duct appears to be partly active and partly by pressure exerted by the ovary walls and possibly other tissues. In *Spadella*, Ghiradelli (1968) indicated that the eggs moved posteriorly in the ovary before entering a very short terminal duct.

Perhaps the greatest source of controversy concerns the structure of the female reproductive organs, and in particular the nature and position of the duct which the egg enters on leaving the ovary. Hertwig (1880) wrote that the ovaries consisted of the ovary or egg tube and the oviduct. Conant (1896), working with *Sagitta hispida*, suggested that there was an oviduct (separate from the sperm duct) lying between the germinal epithelium and the epithelium of the sperm duct. According to his observations, after the eggs were fertilized, they moved through the germinal epithelium into the oviduct, which was a temporary structure.

Stevens (1910) in studying *S. bipunctata*, stated that "both living material and sections show that the sperm duct and oviduct are entirely separate for their whole length, each having its own opening to the exterior" (page 284). She confirmed Conant's (1896) observations concerning the temporary oviduct.

Later authors fell into one or other of the two camps. Bordàs (1920), while working on the same species studied by Stevens, stated that the single tube, although serving as a sperm duct, is principally an oviduct.

Vasiljev (1925) referred to a sperm duct and mentioned elastic fibers running along the length of this duct, which, he said, serve to keep the sperm duct extended. He was unable to find anything resembling a provisional or temporary oviduct, and stated that in *Spadella* the eggs are liberated from the body of the adult animal by a rupture of the body wall occurring close to the entrance of the sperm duct. He also suggested that the accessory fertilization cells and sperm duct degenerated after having fulfilled their role.

Burfield (1927) described the sperm duct and temporary oviduct in detail. He said that the outer duct (oviduct) surrounds the sperm duct which is merely a sperm pouch, having syncytial walls. He did not, however, describe the two openings to the exterior which two such ducts would necessitate.

John (1933), studying *Spadella cephaloptera* and a species of *Sagitta*, confirmed Stevens' (1905, 1910) observations concerning the movement of the eggs, but stated that, in *Spadella*, there was a direct opening between the ovary and the seminal receptacle; however, this orifice was visible only at the time of laying.

John (1943) later had occasion to study *Sagitta* sp. further, and reversed his earlier opinion concerning the presence of two ducts. He stated that there is only one duct, and that what was previously thought to be a second internal duct with syncytial walls was merely "rounded masses of degenerate sperms . . ." (page 71) which would ultimately be reabsorbed.

Dallot (1968) referred to a provisional oviduct, and Ghirardelli (1968), while confirming the observations of Stevens (1910) on *Sagitta bipunctata*, continued "Studies *in vivo* do not allow firm conclusions as to how the eggs reached the outside: only the study of sections obtained from specimens fixed in the various phases of laying has enabled Stevens to describe for the first time the passage of the eggs to the exterior. I have been able to confirm her observations and show the first photographic evidence of the phenomenon" (page 336). Ghirardelli's photographic evidence is of a section however, and shows an egg passing through the crescent: it does not show how the eggs are laid.

While it is possible, and even probable, that differences do indeed exist between one species of chaetognath and another, the above review indicates differences of opinion concerning the same species. Concerning *S. bipunctata*, Bordàs (1920) acknowledged the existence of one duct, whereas Stevens (1905, 1910) was able to see two. In *Spadella cephaloptera*, Vasiljev (1925) mentioned that there is a sperm duct and a temporary oviduct, though he was unable to see the latter, and that the sperm duct is also a temporary structure, while Ghirardelli (1968) indicated that only the oviduct is of a temporary nature. The majority of workers support Conant's (1896) theory that there are two ducts in chaetognaths, and oviduct of a temporary nature, and a permanent sperm duct. However, our observations on *Sagitta hispida* (the species with which Conant, 1896, worked) indicate that there is only one duct, which serves for the admission of the sperm and for the expulsion of the eggs. Apart from our direct visual observations on the direct contact of eggs and sperm within a single duct, we saw extrusion of sperm to the exterior as eggs migrated through the crescent. It seems highly likely that the sperm are being forced into these situations by displacement of the eggs as they enter the same duct occupied by the sperm, although it could be argued that the same effect would be seen in an adjacent tube which was compressed. Sperm which are extruded, however, leave the body via the gonopore (Figs. 1F and 2B) exactly as do the eggs. Further, Reeve and Walter (1972a) showed that insemination confers fertility on one batch of eggs only, subsequent eggs being infertile when laid, if the animal is prevented from being reinseminated. It would seem that a primary advantage of a separate sperm duct would be to store viable sperm for fertilization of ova which had yet to mature.

Stevens (1910) suggested that eggs reached the exterior by their own active movements, crawling along the duct to the gonopore. Ameboid movements were also observed by John (1933) and Ghirardelli (1968). Conant (1896) described contractions of the ovary wall (which surrounds the developing ova, germinal epithelium and duct) as providing the force for the expulsion of eggs in *Sagitta hispida* and Dallot (1968) confirmed this for *S. setosa*. Grassi (1883), Stevens (1910) and Vasiljev (1925) had noted the presence of elastic fibers. Our observations showed that the eggs, which become cylindrically compressed in the duct, are extruded very rapidly by a combination of muscular forces, which includes the body wall musculature as well as the ovary itself.

In *Sagitta hispida*, we observed, as already noted, that eggs leave the body via the single ovarian duct through the gonopore. Regarding other members of the genus, Vasiljev (1925) believed that eggs left the temporary oviduct by being extruded through a rupture in the body wall. From the figures of Ghirardelli (1968) it appears that a temporary opening to the oviduct occurs adjacent to the opening of the sperm duct at the gonopore. In *Spadella*, Ghirardelli (1968) indicated by description and a figure that eggs pass down to the posterior end of the ovary before entering a short temporary oviduct formed by a splitting of the two layers of the wall of the terminal portion of the sperm duct, and exit by a temporary opening to the exterior. John (1933) had previously indicated that the eggs penetrated into the seminal receptacle (the expanded portion of the sperm duct at the gonopore).

Sagitta hispida is a neritic species (Owre, 1960) and is the only planktonic chaetognath which has adapted to the semi-estuarine conditions of Biscayne Bay. The behavioral pattern of attaching eggs presumably assists in maintaining the Biscayne Bay population, since that part of the life cycle during which either the mature adult or the egg is attached is less susceptible to expatriation (an inherent danger of a planktonic existence) into the Florida Current adjacent to Biscayne Bay.

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SUMMARY

1. There is considerable disagreement in the literature concerning the manner in which eggs are fertilized and reach the exterior in chaetognaths, even in studies on the same species. This study makes use of observations on populations of living animals maintained in the laboratory, and of material fixed at known stages of the egg-laying process, and provides photographic evidence to illustrate the points enumerated below.

2. There is a single duct, serving both as an oviduct and sperm duct in the female reproductive system of the chaetognath *Sagitta hispida,* which runs from the gonopore anteriorly along the outer border of each ovary.

3. Following fertilization eggs slowly pass through the germinal epithelium (crescent) through pores much smaller than their own diameter into this duct, and displace the sperm in it.

4. The eggs, which become cylindrically compressed, are extruded very rapidly from the duct to the exterior by a combination of muscular forces, and deposited in a mucous matrix onto a surface to which the animal is attached.

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THE

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ALLOMETRIC STUDIES ON ENERGY RELATIONSHIPS IN THE SPIDER CRAB *LIBINIA EMARGINATA* (LEACH)[1]

Reference: *Biol. Bull.*, **147**: 257–273. (October, 1974)

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Allometry, in the extended sense employed by Gould (1966), is the study of variables exhibiting differential growth. These variables may be morphological (*e.g.*, width/length), physiological (*e.g.*, size/oxygen consumption), or chemical (*e.g.*, size/lipid weight); and the relationships may be dynamic, as in successive measurements made on a growing animal, or they may be static, as in comparisons between non-growing adults.

Changes in the rates of differential growth have been found to accompany maturity in both vertebrates and invertebrates (Teissier, 1931, 1960; Needham, 1942; Brody, 1945; von Bertalanffy, 1957). In decapods, developing maturity is manifested at the molts of prepuberty and puberty (Hartnoll, 1963).

As an animal passes from an immature and actively growing stage to a mature, non-growing and reproducing stage, it is likely that its energy requirements change. Further, if an animal is found in different habitats correlated with successive stages of growth, it is likely that the energy available to it also changes. A succession of growth stages and their associated habitat changes have been termed growth stanzas (Parker and Larkin, 1959). It seemed likely that the energy requirements of a crab would be reflected in the differential growth of variables sensitive to energy requirements, with these differences especially marked when successive growth stanzas were compared.

Now, allometry facilitates the comparison of greatly varying sizes by reducing constant relative growth rates to a power function. The relationship of Y to X is allometric when $Y = bX^a$; where a is the rate of change of Y with respect to X, and b is the intercept, or size of Y when $X = 1$ (Huxley, 1924). Such relative growth rates appear on double-logarithmic plots as straight lines, with changes

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[1] This study formed part of a master's thesis presented at Boston University in 1972. Support was provided by Boston University in the form of teaching fellowships during 1970–1972, and a summer stipend in 1971.

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in rates shown by inflections or discontinuities (Teissier, 1960), as is the case with morphological allometry during the transition from immature to mature stages in Maiid crabs (Hartnoll, 1963).

Hemmingsen (1950) suggested a theoretical limitation of metabolic rate based on the absorptive area of the digestive apparatus. For a crab such a relationship would be shown by similar constants for the slopes of oxygen consumption, stomach capacity, and hepatopancreas weight, this latter being the chief organ of digestion (Vonk, 1960). Such a comparison can be used for other purposes, especially if the sizes of the stomach (or its capacity), and the hepatopancreas (or its lipid content) can be shown to have a maximum likely value at a given size. Such maximum values would be represented by the upper bound of a scattergram of individual points. This is especially valid if the upper bound has the same slope as the overall average. Relative energy flow within a given growth stanza could then be shown by the average size of the organ measured, relative to the regression of the maximum size.

The organ in crabs that is apparently most sensitive to energy flow is the hepatopancreas, with lipids being the most labile constituent, probably reflecting overall energetic demands (Armitage, Buikema and Willems, 1972). This has been extensively studied in relation to the molt cycle by Drach (1939) and Renaud (1949), and has been looked at over growth stages in general in the crayfish *Orconectes nais* (Armitage *et al*, 1972).

This study was therefore designed to examine the allometric relationships of stomach capacity, the adequacy of natural feeding rates, and the size and lipid content of the hepatopancreas; both as related to the state of maturity of the crab reflected by its energetic demands, and the capacity of its habitats to supply these demands. The work done is presented in two major parts. In the first part (morphological allometry) the growth stages are defined by their allometric constants of external morphology, and by their associated habitats. In the second part (physiological allometry), oxygen consumption, stomach capacity and the proportion used, hepatopancreas total and lipid weights, average caloric intake, the cycling rate of stomach contents, and the efficiency of assimilation are treated, and the relative proportions of usage or energy flow computed for the stages previously described.

METHODS

Crabs

Specimens of *Libinia emarginata* (Leach), (Maiidae: Pisinae) the common spider crab of the east coast of the United States, were collected in the shallow waters within a radius of ten miles of Woods Hole, Massachusetts. Immature crabs, from 0.2 to 28 g were usually collected in masses of chlorophytous alga. The crabs clung to this coarse alga and covered themselves with finer filamentous chlorophytes and rhodophytes. Many of these crabs were found in a soft, newly-molted condition, indicating active growth in this habitat. Mature crabs were always collected on relatively barren mud or gravel bottoms, predominantly the former. These larger crabs ranged from 28 to 480 g. A naturalist's dredge was used to collect the immature crabs and an otter trawl for the mature ones. Specimens used for organ analyses were killed and examined within a few hours of collection, others were kept in running seawater tanks and were used for feeding and respiration experiments. Throughout the testing and collecting period of July–September, 1971, the ambient seawater temperature remained between 20–22° C.

Allometry

General. When plotted on double-logarithmic axes, an allometric relation is shown by a straight line having the equation; $\log Y = a \log X + \log b$; where a is the slope of the allometric line and can be measured directly on the plot (Teissier, 1960). Such plots were used initially to identify relationships, but for purposes of statistical comparisons, least squares regressions were computed and F tests of slope (a) and level (b) made between lines. Strictly speaking, none of the regressions can be said to have an independent and a dependent variable, instead they covary with both variables probably under the same influence of differential growth. However, there is a much greater chance of error in measuring lipid weight or oxygen consumption, than there is in measuring total weight or carapace length. Therefore, the standard regression calculation has been made with the more erratic variable being the dependent one. This method has been used for morphological allometry as well, but here the very high correlation coefficients make it unlikely that any serious error is introduced by not using standard allometric methods (for review see Kidwell and Chase, 1967).

In the case of morphological allometry, both a and b are useful in describing growth stages, and both have been reported for many Maiid crabs (*e.g.,* Hartnoll, 1963). In the case of physiological allometry, the comparisons between variables are made on the basis of the slopes (a); testing the similarity of differential growth. The value of b has no meaning when compared here between different variables since it is based on different units and origins. But, when a single variable is compared throughout different growth stages, the value of b represents relative levels of usage in each stage. (Conversely, in this case a has little meaning.)

Morphological. Three identifiable growth stages exist in Maiid crabs; immature, prepubescent, and mature (Hartnoll, 1963). These stages are identified by two important points during the growth of the animal (Teissier, 1935): (1) the molt of prepuberty, where the immature proportions of the crab begin their transformation into those of the adult. This point is marked by an inflection in the allometric line, and is coincident with the first appearance of gonads. Vernet-Cornubert (1958), working with *Pisa tetraodon*, showed that this inflection appears at varying individual sizes. (2) The molt of puberty, or in Maiid crabs, the terminal molt (Carlisle, 1957); at this point the crab is fully mature and the plot of allometric lines exhibits a discontinuity representing the terminal changes in proportions.

The most striking morphological allometries in crabs are those between the length of the male chelipeds (or propodus thereof) and the length of the carapace; and the width of the female abdomen, and the width of the carapace. These measurements were used after the fashion of Teissier (1960) and Hartnoll (1963) to establish the three stages in this crab. Size measurements were made to the nearest 0.5 mm with a caliper.

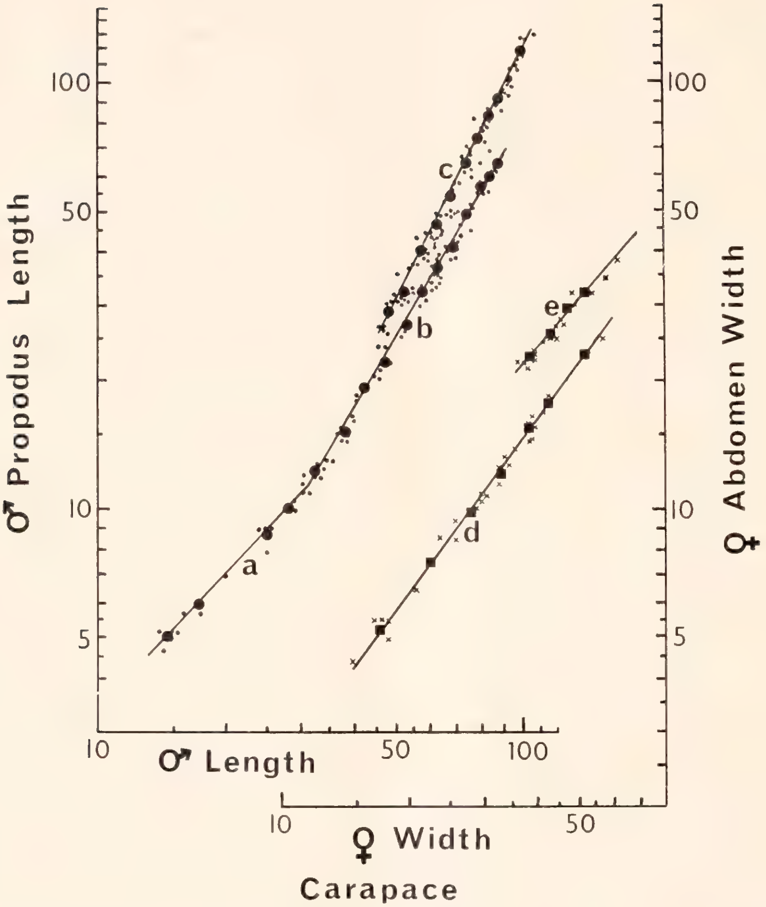

FIGURE 1. Morphological allometry, male propodus/carapace length and female abdomen/carapace widths. Lines are: males, "a" immature, "b" prepubescent, "c" mature; females, "d" immature-prepubescent, "e" mature. Small dots are individual values; large dots are size class averages.

In the case of the female abdomen/carapace allometry, the difference between molting and non-molting stages was apparent upon constructing a scattergram (Figure 1) and they could be collected into immature-prepubescent and mature stages for the computation of allometric constants. A preliminary computation based on size classes (shown in the figure) gave some hint of the prepubescent stage, but this was neither distinguishable in individual crabs nor statistically valid for the purposes of computing constants. The males presented a more difficult case, although the immature and prepubescent sizes were rapidly distinguishable in the scattergram, much of their size ranges intergraded imperceptibly. However, the smallest prepubescent and the largest mature sizes were evident beyond the intergrading region, and the very smallest mature sizes were identified by their mature gonads and also by their having three layers of barnacles, an indication that they had not molted for several years (Hartnoll, 1963). (The subtidal barnacle found on these crabs, *Balanus amphitrite niveus*, sets only once each year according to Ray, 1959). These smallest and largest sizes were used as guides in drawing lines through the uncertain region, separating individual points into prepubescent and mature stages, with ambiguous points arbitrarily divided between the two lines. When all of the points had been assigned to their respective stages, final allometric constants were computed.

Stomach capacity. The maximum stomach capacity was estimated by plotting whole stomach weights (cardiac and pyloric regions together) both as found in newly-collected specimens and in some fed to satiety. Feeding was necessary in large crabs because they were never collected with full stomachs. After weighing whole, the stomachs were flushed and blotted and the empty weight recorded. Separate regression lines were computed for the maxium points, and for the empty points, the difference between these lines at a given size being taken as the maximum capacity. For purposes of computation, the specific gravity of stomach contents was considered to be unity so that one gram was equivalent to one cc of contained food.

Oxygen consumption. This was measured in closed containers, the dissolved oxygen not being allowed to decrease to less than $\frac{3}{4}$ of the ambient level (Stroganov, 1964). Measurements were obtained for the entire size range of *Libinia*, from newly-released zoeae, to the largest (450 g) mature males (Rathbun, 1925). Such a millionfold span of weight decreases the erratic value for a that can be found when using small ranges of weight (Hemmingsen, 1960). Large crabs were measured singly, crabs less than 5 g were measured in groups, and about a thousand zoeae were measured simultaneously. The length of the experiments varied from half an hour in the largest crabs (measured in a submerged wash basin with a plexiglas lid), to several hours in the smallest crabs (measured in submerged plexiglas boxes). These measurements were made only upon freshly-collected, non-molting specimens (23), and were considered to represent the routine rate (Bayne, 1973).

Hepatopancreas. This was weighed wet without draining, and portions of tissue frozen for later lipid analysis by the chloroform-methanol method of Folch, Lees and Sloane-Stanley (1957). Subsequent calculations were based on wet weight.

Stomach contents. The contents removed in the stomach capacity measurements were recorded as rough percentages in four categories: (1) Detritus, the flocculent upper 2–3 cm layer of muddy bottoms (Sanders, 1960). Because of the variability in published values for the organic content of this material, two samples were ashed giving an average organic (burnable) content of 1.83%. Figuring 4,000 cal/g (carbohydrate) this amounts to 73 cal/cc. (2) Algae, mostly fine filamentous rhodophyta and cholorophyta; as determined by ashing, their organic content averaged 7.5% of the wet weight. At 4,000 cal/g (Odum, 1963) this is an average of 300 cal/cc. (3) Invertebrates, primarily pelecypods and marine worms, indicated by valves and setae. An average dry weight of 20% was assumed, and multiplied by 5,700 cal/g (Brody, 1945) as protein, gives 1,140 cal/cc. (4) Flesh, this last general type consisted of small squid and fish and was figured at the same caloric value as (3). These percentages, multiplied by the weight of the contents gave the caloric value for each stomach examined.

The total weight of the contents was compared to the maximum capacity of the stomach, indicating the percentage of capacity used.

Feeding experiments. Four sets of feeding experiments were made having durations of 9–13 days. Crabs previously starved for up to two weeks were fed each day, uneaten food from the previous day being removed and weighed. The maximum rates of consumption were usually realized during the first day of the test, consumption declining rather steadily during succeeding days. Because they occur in groups under natural conditions, the crabs were fed in groups and the rates obtained are averages. These averages are expressed as multiples of the maximum stomach capacity (stomachsfull) eaten per day.

Assimilation efficiency. This was measured during the four sets of feeding experiments. Faeces were collected several times each day to prevent their possible consumption by the crabs (never observed). The crabs defecated immediately upon their first feeding, therefore the faeces produced on a given day might be the result of an earlier feeding. Faeces output, like food consumption, declined from a maximum during the first days, and the two rates were in proportion, but with the faeces output lagging behind food consumption by 2–3 days. Because of this lag, overall assimilation efficiencies were computed for each experiment on the basis of the total dry weights of food consumed and faeces produced.

Table I

Allometric constants for the equation: log $Y = a\log X + \log b$; and a = the slope of the line, b is the intercept when $X = 1$; R = correlation coefficient

Stage	*a*	*b*	R
(a) Morphological allometry			
Males: Carapace/propodus lengths			
Immature	1.08	−0.56	0.9934
Known prepubescent	1.61	−1.36	0.9660
All prepubescent	1.72	−1.53	0.9915
Known mature	1.98	−1.89	0.9956
All mature	1.95	−1.85	0.9830
Females: Carapace/abdomen widths			
Immature	1.28	−0.86	0.9898
Prepubescent	1.35	−0.95	0.9854
Mature	1.12	−0.42	0.9791
(b) Physiological allometry			
Stomach fullness/live weight			
All immature full	0.80	−1.37	0.7901
All immature empty	0.97	−2.00	0.9058
Mature female full	1.01	−1.71	0.8190
Mature female empty	1.31	−2.77	0.9234
Mature male full	0.51	−0.94	0.8108
Mature male empty	0.78	−1.87	0.9096

TABLE II

F tests of regression lines showing; f values for a and b (slope and level), degrees of freedom, and level of significance (1%, 5%, X = not significant)

Pair of lines tested	*a* (slope)			*b* (level)		
	f	d.f.	Sig	f	d.f.	Sig
(Male propodus)						
Immature/known prepubescent	25.4	1/35	1	8.77	1/36	1
Known prepubescent/known mature	8.79	1/51	1	33.4	1/52	1
All prepubescent/all mature	24.5	1/183	1	237	1/184	1
(Female abdomen)						
Immature/prepubescent	0.646	1/24	X	0.891	1/25	X
Prepubescent/mature	7.48	1/31	5	539	1/32	1
Immature-prepubescent/mature	3.47	2/42	5	274	2/44	5
(Stomach weights)						
All maximum/all empty	0.491	1/11	X	2376	1/12	5
Immature as found/immature empty	0.543	1/22	X	6.00	1/23	5
Mature female as found/m.f. empty	5.03	1/36	5	72.9	1/37	5
Mature male as found/m.m. empty	1.00	1/18	X	84.3	1/19	5
(Hepatopancreas)						
Immature wet wt/all mature wet wt	0.039	1/56	X	12.7	1/57	5
Imm. lipid wt/all mature lipid wt	0.162	1/56	X	9.89	1/57	5
Routine O_2/imm. hepato. wet wt	1.06	1/44	X	153	1/45	7
Routine O_2/imm. lipid wt	0.267	1/47	X	—	—	—
Routine O_2/all stomach empty wt	0.083	1/63	X	—	—	—

RESULTS

Morphological allometry

The plots of male propodus/carapace lengths, and female abdomen/carapace widths are given in Figure 1, where both individual points (small dots) and size class averages (large dots) are shown. For purposes of computation the males were divided into five categories: (1) immature; (2) known prepubescent (this excluded large doubtful cases); (3) known mature (small barnacled sizes and large crabs with extreme propodus lengths); (4) all possible prepubescent; (5) all possible mature. The allometric constants (Table Ia) were compared by F tests (Table II). The lines including the doubtful points were indistinguishable from those formed only by known points, therefore these all inclusive lines were used. Male immature, prepubescent, and mature stages were all significantly different in slope and level. The inflection at the molt of prepuberty occurs at the average carapace length of 31.5 mm, and is evident in the figure from the difference in slope between lines "a" (immature) and "b" (prepubescent). The mature crabs (line "c") range in length from 47.0 to 106.0 mm, their individual points making a line distinct from that of the prepubescent crabs, which are found up to 87.0 mm long.

Female crabs were divided into three categories for computation: (1) immature; (2) prepubescent; (3) mature. The molt of prepuberty was not significant (Table II), and immature and prepubescent females were treated together. This combined stage (line "d") was found up to the carapace width of 43.0 mm. Mature females were found over the width range of 37.0 to 62.0 mm (line "e"), and were significantly different from the immature line. Expressed as carapace lengths for comparison to the males, these figures are 52.5 mm for line "d," and 42.0 to 77.5 mm for line "e."

The most distinct differences in the physiological constants were subsequently found between the molting (immature and prepubescent) and non-molting (mature) crabs, therefore immature and prepubescent crabs of both sexes are treated together in the following comparisons, with mature females and mature males treated separately for a total of three stages, equivalent to two major growth stanzas within each sex. The first stanza is immature, actively growing and found in an abundance of algal food. The second is mature, reproducing, non-growing, and found on relatively barren mud bottoms. (This second stanza might be termed a "life" stanza because there is no growth.) The planktonic larvae of a crab would form a third major growth stanza.

Physiological allometry

Oxygen consumption measurements gave an average routine rate of 4.7 ml O_2/hr for a crab of 123 g fresh weight, similar to other published values. Vernberg (1956) gives 3.2 ml O_2/hr for this weight of *L. emarginata*, and Zeuthen (1953) gives a general value for crustacea equivalent to 3.7 ml O_2/hr at this weight. The values found in the present study may be termed a lower range of the excited rate due to measuring after handling (Aldrich, 1974a). Odd, high values, probably representing elevated rates due to biological rhythms (Ansell, 1973; Aldrich, 1974b) were excluded from the calculation. The allometric constants for ml O_2/g/hr were: $a = 0.81$, $b = 0.89$, $R = 0.9950$. The slope (a) is in virtual agreement with the a of 0.80 found by Zeuthen (1953). The average daily caloric cost of maintenance was computed at 4.83 cal/ml O_2 (Brody, 1945) for a basal rate (the very lowest range of points in the overall regression) 80% of the routine rate.

Stomach weights are given for individual crabs in Figure 2. The regression of maximum full weights has the constants: $a = 0.80$, $b = -1.15$, $R = 0.9950$; and the regression of all empty stomachs has the constants: $a = 0.80$, $b = -1.89$, $R = 0.9566$. Thus the full and empty stomach weights bear a constant relationship to one another over a hundredfold range of body weight. Therefore the capacity of the stomach has a constant allometric relationship to the weight of the crab. The slope of this capacity regression is virtually the same as that of the routine metabolism (an F test showed no significant difference). Although this does not prove that metabolic rate is dependent upon the capacity of the stomach, it does indicate that these relationships are similarly constant with increasing weight, suggesting a functional correlation.

The percentage of the maximum stomach capacity used is not the same in the three stages (Figure 2). Within each stage there is a significant difference between the levels of "as found" and empty stomach weights (Table II, b values). Immature-prepubescent, and mature females appear to use almost the maximum capacity, but large mature males seem to use a decreasing proportion as they increase in size. This is reflected in the significantly different slopes of the male "as found" and empty regressions (Table II), a difference not found in other stages and implying the constancy of empty weights, but the relative decrease of

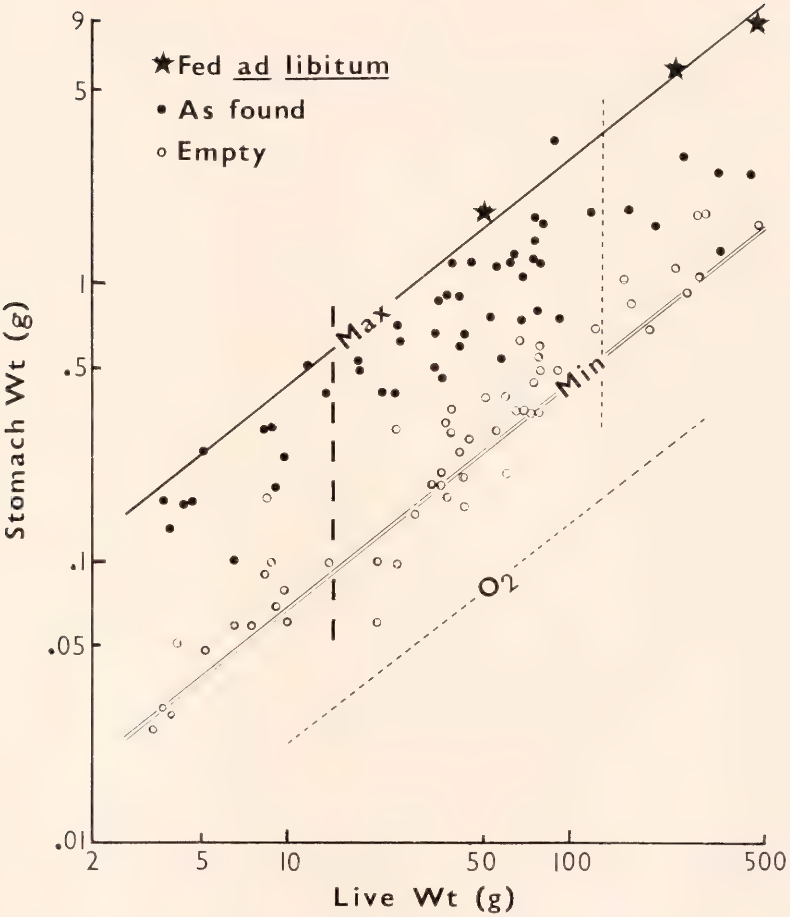

FIGURE 2. Stomach capacity and weight of contents found in collected crabs with maximum capacity = Max. line – Min. line at any given crab weight. Size ranges are: immature crabs to the left of the heavy dashed line; largest mature males to the right of the light dashed line; mature females and small mature males between the two dashed lines. Individual values both as found and when emptied are given. The regression of oxygen consumption is shown for comparison of slope, and is parallel to the regression of stomach capacity.

fullness with increasing size. Note the different empty stomach slopes (*a* in Table Ib) for the three stages and how their overall combination makes a "regular" allometric line parallel to that of the routine oxygen consumption.

The relationships of hepatopancreas total and lipid weights to total live weight are shown in Figure 3. Here immature-prepubescent crabs, as well as very large male crabs that have recently molted to maturity (Points "N") fall on the same regression line of hepatopancreas wet weight. The constants of this line are: $a = 0.82$, $b = -0.84$, $R = 0.9280$; therefore it is parallel to the regression of routine metabolism (an F test showed no significant difference in slopes, see Table II). The combined regression of mature male and female hepatopancreas wet weight has the constants: $a = 0.86$, $b = -1.16$, R = 0.8150 and is not significantly different in slope from the previous regression, but is different in level. Thus the weight of the hepatopancreas follows the same allometric formula (slope of maximum weight) as the stomach capacity and routine metabolism, and again larger (mature) crabs have lower levels (b) of utilization of a physiological capacity. (That this drop in proportion is not due simply to the larger sizes of mature crabs is shown by the points "N" for very large newly-molted crabs.)

The total weight of lipid contained in the hepatopancreas follows an analogous pattern. Here the constants are: Immature-prepubescent, $a = 0.98$, $b = -2.08$, R = 0.644; all mature, $a = 1.16$, $b = 2.98$, R = 0.5758. F tests showed that these two lines are insignificantly different in slope, but again significantly different in level (Table II). Due to the great variability in individual values, an F test showed no difference between the immature-prepubescent slope and that of the routine metabolism.

The lower level of utilization of physiological capacity in mature crabs is again suggested by the caloric value of stomach contents (Figure 4). Here the caloric value decreases relative to the cost of maintenance as the size of the crab increases,

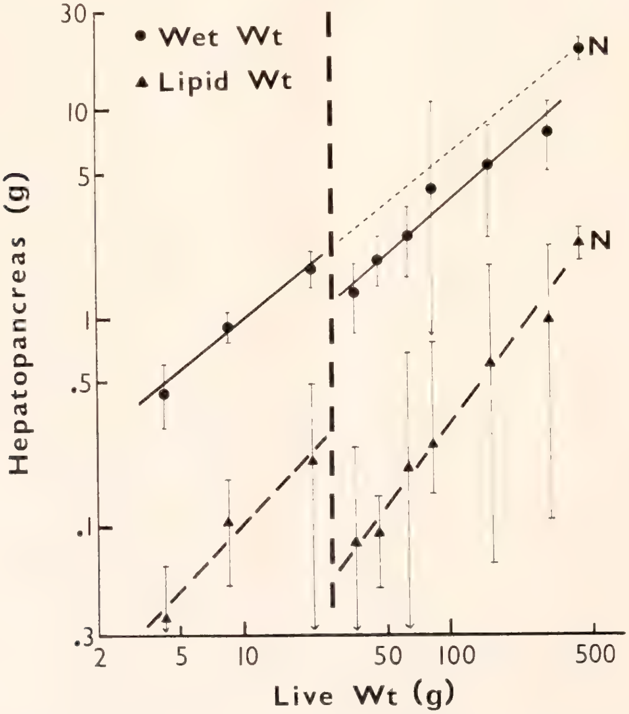

FIGURE 3. Hepatopancreas wet (total) weight (upper solid lines), and lipid weight (lower dashed lines). Data presented as size classes with standard deviations shown by the vertical bars. Crabs to the left of the vertical dashed line are immature-prepubescent, those to the right are mature (both males and females), the points "N" are recently molted males.

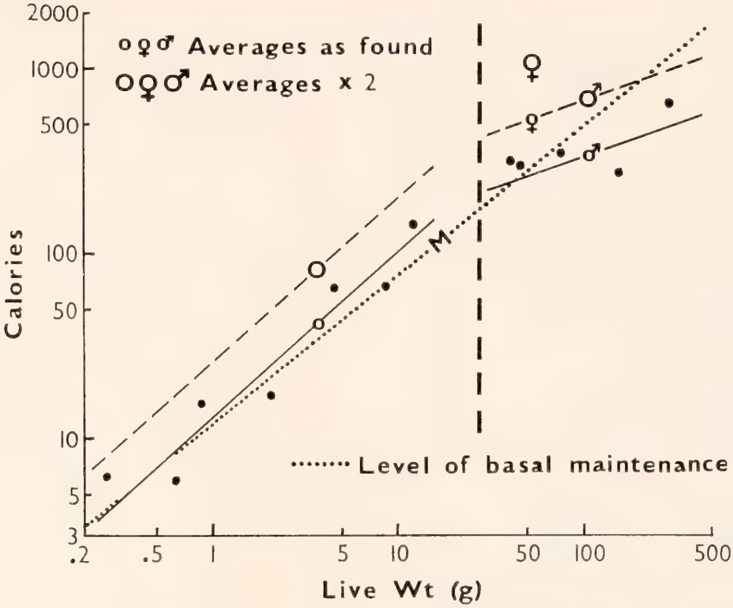

FIGURE 4. Caloric value of stomach contents found in collected crabs, contrasted with the caloric cost of basal maintenance. Crabs to the left of the vertical dashed line are immature-prepubescent, those to the right are mature. Data presented as size class averages (dots) and means (small symbols) for immature-prepubescent, mature females (mean only), and mature males. The effect of the cycling rate is shown by doubling the caloric values (large symbols and dashed regression lines).

especially in mature males. Again, immature crabs maintain a balance close to the maintenance cost, the regression of caloric values following the same slope as metabolism. Mature females also have a balance close to the maintenance cost. (The cost of monthly reproduction is less than that of rapid immature growth, Aldrich, 1972.) These trends suggested by the stomach contents are not as clear as the preceding ones due to the extreme variability in both the amount and caloric value of the contents (Table III), and the presentation is made without statistical significance (according to correlation coefficients). However, the trends agree with those previously found, the low caloric values for the mature males are especially marked, suggesting a real underutilization of stomach capacity.

The stomach content findings are complicated by the cycling rate, or multiples of stomach capacity (stomachsfull) that can be processed in a 24 hour period. In four sets of feeding experiments the maximum consumption in 24 hours averaged 1.95 stomachsfull (Table, IV). This means that the estimated maximum stomach capacity could be processed twice a day, or the cycling rate is such that any given contents could be digested within 12 hours. These figures were obtained with cleaned fish, mussels, and algae, all readily digestible. An earlier experiment (No. 5 in Table IV) where the crabs fed on starfish gave much the same result with this relatively undigestible food.

TABLE III

Percentage fullness (% of max. capacity) and stomach contents of crabs examined within 2 hours of collection

Size class (grams)	No.	Ave. % fullness	% Fullness acc. to food types			
			Detritus	Alga	Inverts.	Flesh
			Immature males and females			
0.00–0.33	3	100		100		
0.34–0.67	2	50		50		
0.68–1.00	2	100		100		
1.1–3.3	8	56		50		6
3.4–6.7	8	75		65	10	
6.8–10	4	70		70		
11–33	1	100		100		
			Mature females			
34–122	22	38	9	9	11	9
			Prepubescent males			
14–74	5	46	4	23	19	
			Mature males			
33–67	7	44	15	12	7	10
68–100	7	51	20	23		8
101–200	7	19	7	7	5	
201–500	5	14	4	10		

The assimilation efficiency averaged 96% (Table IV), so high that it was considered unity for purposes of calculations. There was no real difference in efficiencies whether the crabs were digesting animal or vegetable food. Such

TABLE IV

Feeding experiments showing average weight and number of crabs, food given (cleaned fish, mussel flesh, algae, and whole starfish), multiples of stomach capacity (stomachsfull) processed within 24 hours, and overall assimilation efficiencies

Exp. No.	Ave. Wt. ± S.D.	No. Crabs	Food	Multiple	Assim. Efficiency
1	5.8 ± 3.41	5	Fish, *Stenotomus*	1.53	96.5%
2	37.2 ± 6.59	10	Mussel, *Mytilus*	1.90	96.7%
3	45.0 ± 13.5	25	Fish, *Stenotomus*	2.05	99.3%
4	58.7 ± 10.1	8	Alga, *Codium*	2.31	95.0%
(5)	144 ± 169	16	Starfish, *Asterias*	(1.65)	—

high efficiencies are not uncommon in arthropods consuming animal food (Lawton, 1970, 1971).

Discussion

As indicated by the scattergram (Figure 1), there is a considerable range of mature sizes in both males and females. For males, this range requires three molts at an average increase factor of 1.26 (Dyar's Law; Teissier, 1960). Two of these molts are optional in the sense that some prepubescent crabs exist in ever increasing sizes but still have not reached maturity as defined by the inability to undergo further molts. Because some of these larger prepubescent crabs appear to have mature gonads, "prepuberty" for them may include animals sexually mature before reaching their terminal anecdysis (Hartnoll, 1963). Some very large prepubescent males were kept in the laboratory tanks and they all molted in the autumn, whereas no mature crabs of any size ever molted. The consequence of the optical molts is a 16-fold weight range for mature, non-molting male crabs. This weight range is similar to that found for many other Maiid crabs (Hartnoll, 1963; Vernet-Cornubert, 1958), but was not anticipated since it is "common knowledge" that only the largest males are the mature sizes. Some of the smallest mature males were kept together with mature females and they actively pursued and mated with them, giving a behavioral confirmation of the status suggested by their allometry.

The lesser range of female mature sizes requires two molts, of which one would be optional. Smaller mature females were often collected bearing eggs, as well as the commoner ovigerous middle range, but none of the very largest mature females were collected with eggs. The reproductive status of these largest females crabs is therefore unclear.

Thus the morphological allometry made possible the recognition of this great range of mature sizes, leading apparently to quite different energetic requirements within the mature stage. Small crabs appear to live for several years when mature, as evidenced by their several layers of barnacles. Such crabs were usually collected in rocky habitats where food may have been more abundant than on the mud flats where the largest mature crabs were found. Large crabs were never found with more than a few scattered barnacles, an indication that they may not have lived as long as the small males. Two reproductive strategies may be available to Maiid crabs then; small size (and small numbers of larvae in the case of females) coupled with several reproductive years, and large size coupled with a shorter reproductive period. Although females will accept the smaller males as mates, the larger males have a distinct competitive advantage, when kept together in tanks during these experiments, they merely picked up and cast aside their smaller rivals.

Three major points recur in the physiological variables. First, the maximum capacity of the stomach, the maximum hepatopancreas weight, and the metabolic rate all follow the same slope (a) thus exhibiting similar physiological relationships at the maximum values. Secondly, regularly molting crabs exhibit high proportional usage of digestive capacity and hepatopancreas storage capacity as shown by their relatively high values of b (level). Thirdly, the mature stages (non-molting) are functioning at less than the maximum physiological capacity for these factors. The weight and lipid content of the hepatopancreas, as well as the portion of stomach capacity used are lower than in the molting stage. This lower level of usage is reflected in their *b* values.

The calculated daily levels of caloric intake based on stomach contents could be doubled by assuming that these levels were processed twice daily, and that there was no correction for undigested food. This is probably an overestimate for the larger crabs since there would be no advantage in consuming less than the maximum possible in the food-poor habitat of the muddy bottoms. In this case the stomach contents may represent the actual daily consumption. However, doubling the caloric value of the stomach contents may be a good estimate for the growing stage. The great rate of growth (up to monthly doubling in weight) may require as much food as they can process and their algal habitat would supply it without limit.

Whatever the rate of processing stomach contents may have to do with their average "as found" level, the mature males appear to use less of their capacity as their size increases. Even doubling the caloric level of the contents leaves the largest males with an intake less than the minimum required for basal metabolism, as is shown in Figure 4 by the dashed lines through the average caloric values X 2. Further, mature females found in the same habitat are relatively better fed than the mature males. In the summer, females must bear the cost of monthly reproduction (Hinsch, 1968, 1972) and require more food than males, yet some specimens had up to three layers of barnacles, apparently indicating several years in the mature stage.

Although mature females may be relatively better feeders than males, it could be that mature males do not have an "inclination" to eat at a sufficient rate for long survival. Perhaps such large crabs, those that have passed through the optional molts, represent a specialized reproductive stage like spawning male salmon that never feed. This idea is strengthened by the large crabs not being found with layers of barnacles, suggesting that they do not live long. There may be a general reduction in feeding levels in mature crabs, at least during certain stages of reproduction. Pearson (1908) mentions that berried *Cancer pagurus* "feed very little," and Carlisle (1957) described the "heaps" formed by molting and mating *Mya squinado,* one persisting for two months. Presumably the crabs did not feed while in this stationary heap. (In this context, the author remembers as a little boy finding a heap of *Libinia*, a discovery rapidly abandoned.) The very small non-molting mature sizes were not examined separately and it would be instructive to see if there is a marked drop in their *b* values corresponding to that found in large mature sizes. This would show if there is a size effect within the mature stage or whether *b* values simply drop with the cessation of growth.

In all, the differences in *b* values between immature-prepubescent crabs (the first growth stanza) and mature ones (the second stanza) are significant and consistent throughout the variables compared. Because Maiid crabs do not molt when mature, this stanza is quite distinct and the size of the hepatopancreas may be a better indicator of relative energy demand than in brachyrhynchous crabs where molting continues through most of the reproductive life. One such brachyrhynchous crab, *Carcinus maenas*, exhibits seasonal fluctuations in metabolites stored in the hepatopancreas, but has no obvious storage of material in preparation for ecdysis, there being a marked variation between individuals in the later molt stages (Heath and Barnes, 1970). Seasonal fluctuations were not measured in *Libinia* and their effects may be important, but the molting cycle will not complicate the interpretation as it would in another brachyrhynchous crab, *Cancer pagurus* (Renuad, 1949). Whether or not the hepatopancreas does indicate relative energy demands, the level of stomach contents must indicate the rate of energy intake and this level is also shown to decrease in mature *Libinia.* The habitats defining the immature-prepubescent, and mature growth stanzas are different, and the rate of energy supply in the habitat of the mature crabs must be quite limited. As the points "N" in Figure 4 imply, the condition of newly-molted but very large mature crabs appears to be similar to a normal immature growing crab. However, the reserves are not maintained during subsequent life in the mature stanza. The capacity of the crab as a system probably remains unaltered, as is suggested by the similar *a's* for maximum stomach capacity and hepatopancreas weight. Thus the mature stage of this crab appears to be an underutilized end point of a system designed for the growth of the immature stage.

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SUMMARY

1. Allometric plots were used to separate *Libinia emarginata* into three male growth stages; immature, prepubescent, and mature; and two female stages; immature-prepubescent, and mature.

2. The existence of two optional prepubescent molts in males allows a 2.75-fold variation in mature (non-molting) length; and one optional molt in females allows a 1.75-fold variation in mature length.

3. Because of the very great size range in mature crabs (especially males), there are apparently two reproductive strategies; small mature size and several reproductive years, and large size with one reproductive year.

4. The regression of routine oxygen consumption, measured over the entire size range of this crab, exhibits virtually the same slope (0.81) as found by Zeuthen (1953) for crustacea in general.

5. The maximum capacity of the stomach, and the maximum hepatopancreas wet weight follow the same allometric relation (slope or a) as the oxygen consumption, in agreement with the idea that metabolic rate is reflected in the size of the digestive apparatus (Hemmingsen, 1950).

6. The actual weight of stomach contents, and the wet and lipid weights of the hepatopancreas (level or b) are not fixed and reflect relative energetic demands or availability.

7. During the stages undergoing regular molts (immature and prepubescent) these variables exhibit high level implying the maximal use of physiological capacity.

8. At the stage of maturity, these levels decrease relative to the growing stages, implying the underutilization of this capacity.

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ROLES OF OXYGEN AND CARBON DIOXIDE IN THE CONTROL OF SPIRACULAR FUNCTION IN CECROPIA PUPAE

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In most insects spiracular valve movements are controlled by oxygen and carbon dioxide (Beckel and Schneiderman, 1957; Schneiderman, 1960; Levy and Schneiderman, 1966a, 1966b). When the intratracheal concentration of carbon dioxide is high or that of oxygen is low, the spiracular valves open; when the intratracheal concentration of oxygen is high, they close. However, the sites at which these gases act have never been demonstrated. The central nervous system is surely involved (Miller, 1966) as are the spiracles themselves. This report identifies the targets of oxygen and carbon dioxide in diapausing pupae of the Cecropia moth, discusses the interaction of oxygen and carbon dioxide in controlling spiracular behavior, and analyzes several aspects of the nervous control of the spiracular closer muscle.

Materials and Methods

Diapausing pupae of the silkworm *Hyalophora cecropia* were used. They were stored in their cocoons at 2° C and 90% R.H. until they were prepared for experiments as follows: their abdomens were immobilized with strips of paraffin (Schneiderman and Williams, 1955) to prevent internal movements from damaging the ganglion preparation, and external telescoping movements from obscuring the spiracles of movable segments. The brain was removed to insure permanent diapause (Williams, 1946). Pupae then were allowed to recover for at least two weeks at 25° C and 50% R. H.

Two days before an experiment was begun the pupa was provided with an "inlet" and an "outlet" cannula (Fig. 1) which enabled us to perfuse the entire tracheal system, including the tracheae serving the spiracular muscles ("spiracular' flow). Cannulae were 8 cm lengths of polyethylene tubing (0.97 o.d., 0.58 mm i.d. (PE-50); or 0.61 mm o.d., 0.28 mm i.d. (PE-10)) (Intramedic tubing, Clay-Adams Company, New York). Each cannula was inserted past the spiracular valve (Schneiderman and Schechter, 1966; Brockway and Schneiderman, 1967). The cannulae were checked for leaks as described by Brockway and Schneiderman (1967). The experimental gas was introduced into the tracheal system via the second or third right abdominal spiracle, and excess gas escaped via either the fifth or sixth left abdominal spiracle.

The fourth abdominal ganglion was selected for neural and tracheal surgery since it is located conveniently in a non-collapsible segment, the spiracles of which are not obscured by overlying pupal wingpads. The spiracle on the right side of that segment was exposed and observed in all experiments. In addition the third or fifth right abdominal spiracle or both were exposed and observed as control spiracles.

The condition of the exposed spiracular valves was checked daily by probing the pupa gently on the cuticle in the area of the exposed spiracles; this maneuver caused properly functioning valves to open. In air, valves of a normal pupa pulsate most of the time, that is, they move but do not open. At the beginning of each experiment moist air was perfused through the pupa's tracheal system; if the valves failed to pulsate, the pupa was discarded.

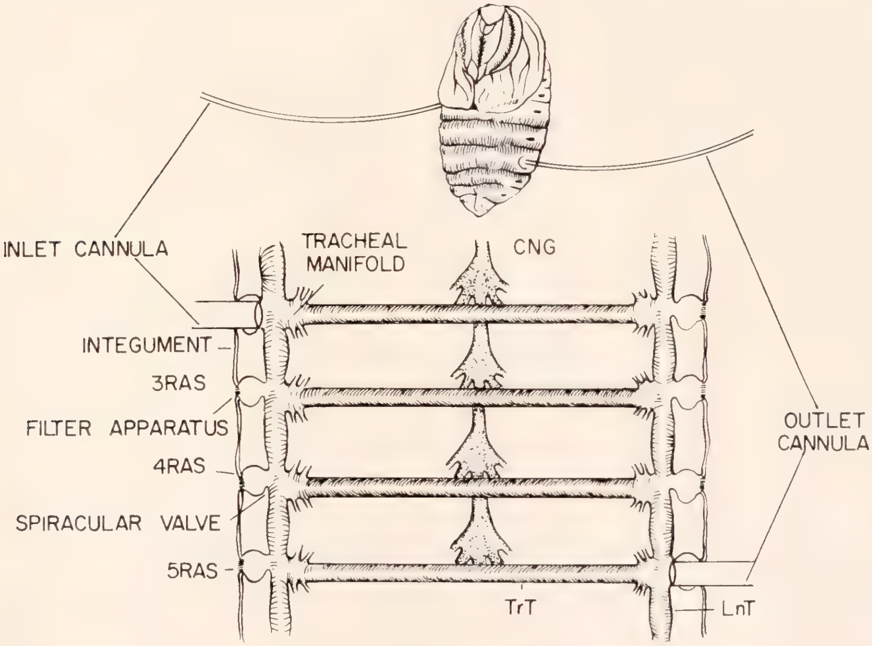

FIGURE 1. Arrangement of cannulae for spiracular flow. The upper part of the diagram shows a pupa with the inlet and the outlet cannula in position; the lower part of the figure shows a portion of the tracheal system prefused via this arrangement. The inlet cannula (2RAS, above 3RAS) was connected to the perfusion pump, and the outlet cannula (5LAS, opposite 5RAS) was open to the atmosphere. Gas entering the inlet cannula flowed throughout the tracheal system; excess gas escaped via the outlet cannula; 3RAS, 4RAS, 5RAS—third, fourth, fifth right abdominal spiracle; CNG—ganglion; TrT—transverse trachea; LnT—longitudinal trachea.

Animals were mounted ventral side up in modeling clay for observations. Experiments were performed at 22–25° C and 40–50% R.H. During the recovery intervals between gas perfusions the pupa was kept in a water-saturated environment.

Anatomy of the spiracle

The spiracular apparatus was described in detail by Beckel (1958) (Fig. 2). It consists of a membranous spiracular valve, a chitinous frame which acts as a lever, and the spiracular closer muscle which is opposed by an elastic ligament and is innervated by a single spiracular nerve (Fig. 3).

Recording spiracular valve movements

The method described by Schneiderman (1956, 1960), in which the leading edge of the spiracular valve is followed in an ocular micrometer, was used for recording valve movements. A Grass FT 03 force displacement transducer and polygraph (Grass Instrument Company, Quincy, Massachusetts) were substituted for the lever-pen system. All except the most rapid and frequent valve movements could be followed and recorded almost as soon as they occurred. It was possible to record the movements of only one valve at a time. However, all spiracular valves engage in the same mode of behavior even though they are not synchronous (Van der Kloot, 1963; Brockway and Schneiderman, 1967), *i.e.*, they constrict, flutter, or open fully. Hence the behavior of the observed valve is qualitively like that of the others. A dissecting microscope permitted viewing of several valves in turn at 30 × without disturbing the preparation. Consecutive recordings lasting 10 seconds to 3 minutes each were made when observations of two or more valves were required.

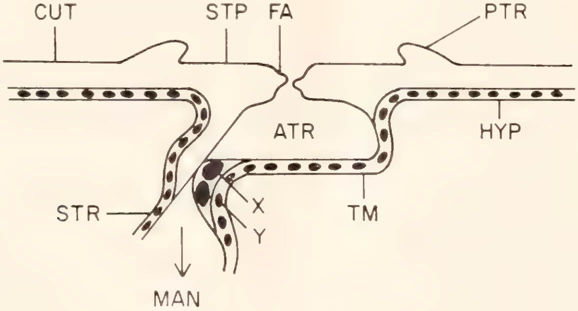

FIGURE 2. Frontal section through the spiracular region (redrawn from Beckel, 1958). The closing bars push the tracheal membrane against the closing bow, thus closing the spiracular opening as in this drawing; ATR—atrium; CUT—cuticle; FA—filter apparatus; HYP—hypodermis; MAN—manifold; PTR—peritreme; STP—stigmal plate; STR—spiracular trachea; X, Y—dorsal lateral and dorsal median closing bar, respectively.

Gas perfusions

Gas mixtures were made up in small cyclinders or directly in a 50 ml syringe from commercially available cylinders of oxygen, carbon dioxide, and nitrogen The syringe was equipped with a three-way Luer-Lok MS 02 valve which permitted direct filling of the syringe from cylinder lines at approximately atmospheric pressure, and minimized the possibility of contaminating the contents of the syringe with external air. The syringe could be refilled in less than one minute when long perfusions were necessary. Gas analyses accurate to ±1% were performed, using a Model E2 paramagnetic oxygen analyzer and a Model 215 infrared carbon dioxide analyzer (Beckman Instruments, Inc., Fullerton, California). As recommended by the manufacturer, neoprene tubing was used for cylinder lines and Viton-A vacuum tubing (Bearings, Inc., Cleveland, Ohio) connections were used with the analyzers. These materials are impermeable to the gases used in these experiments. A hypodermic needle was attachd to the syringe valve; a 50 cm length of polyethylene tubing the same diameter as the inlet cannula of the pupa connected the needle with the cannula. The plunger of the syringe was driven at a contant predetermined rate by a motor. Two syringes were used when two gas mixtures were perfused simultaneously.

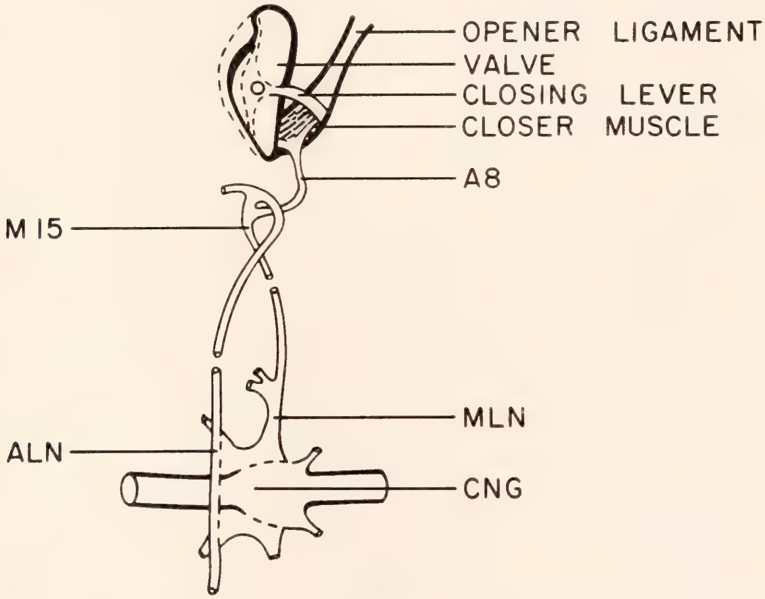

FIGURE 3. Innervation of an abdominal spiracular closer muscle (redrawn from Beckel, 1958). CNG—ganglion; ALN—anterior lateral nerve; MLN—mid-lateral nerve; A8—branch of ALN innervating the muscle; M15—branch of MLN innervating the muscle.

Animals were perfused at a rate of 360 μl/min (occasionally 1430 μl/min) for at least 20 minutes or until a definite unchanging pattern of spiracular response had been established. A 20-minute recovery period was allowed between exposures to different gases although normal behavior of spiracular valves, assuming that behavior in ambient air is "normal," was resumed usually within 10 minutes after the gas flow had been terminated.

As a routine check on the initial condition of the pupa, and to determine the pattern of spiracular valve activity, the following procedure was used: The tracheal system of each of 59 pupae was perfused before the experiment began with at least 5 of the following: 2, 3, 5, 10, 21 (air), or 50% O_2 (balance N_2 and CO_2). Since the tracheal system normally contains at least 3% CO_2 (Levy and Schneiderman, 1966b), 3 or 4% CO_2 was added. Similar results were obtained whether the concentration of oxygen was selected at random or progressively increased from 2 or 3% to 21 or 50%. Records of valve responses by each pupa to different gas mixtures were used as controls for subsequent experimental records of that same pupa.

Perfusing the tracheae of an abdominal ganglion

To provide the fourth abdominal ganglion with its own gas supply, separate from that of the rest of the pupa, the following procedures were used:

An 8 mm square of integument overlying portions of the third, fourth, and fifth abdominal segments was removed. A few crystals of a 1 : 1 : 1 mixture of phenylthiourea, streptomycin sulfate, and penicillin were placed in the wound. Any lost hemolymph was replaced with insect Ringer (Ephrussi and Beadle, 1936). The same gas mixtures that were used before surgery were perfused through the pupa's tracheal system immediately after surgery. Thus the spiracular valve responses of a pupa to various oxygen concentrations just before and just after injury could be compared.

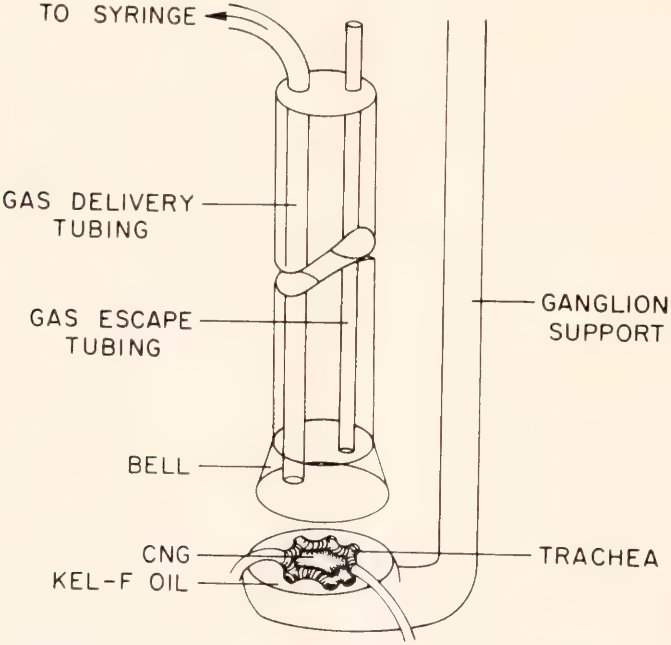

FIGURE 4. Assembly for supporting the fourth abdominal ganglion and perfusing it with the ganglionic flow. The bowl of the ladle was filled with Kel-F oil to retard desiccation and to prevent the ganglion from sticking. The gas escape tubing permitted excess gas to escape and prevented the Kel-F oil from bubbling; CNG—ganglion.

Following this second set of perfusions, all fat body was removed from the vicinity of the third and fourth abdominal ganglia. This procedure did not interrupt the tracheal supply to the spiracular closer muscle of the fourth spiracle or that of its adjacent control spiracles. The intact fourth abdominal ganglion was supported from its dorsal side in the bowl of a "ladle" which was inserted beneath the ganglion with a micromanipulator (Fig. 4). A second micromanipulator positioned the gas delivery bell directly over the ganglion. The bell completely enclosed the ganglion resting on its support but did not compress the nerves or tracheae supplying the ganglion. The entire assembly dipped into a drop of Kel-F high polymer oil (Minnesota Mining and Manufacturing Company, St. Paul, Minnesota) so that no external gases contaminated the experimental gas flow. Thus the fourth abdominal ganglion could receive its own separate, localized gas flow via this arrangement ("ganglionic" flow) while a second gas could be perfused simultaneously through the animal's tracheal system (spiracular flow). The third abdominal ganglion served as a control.

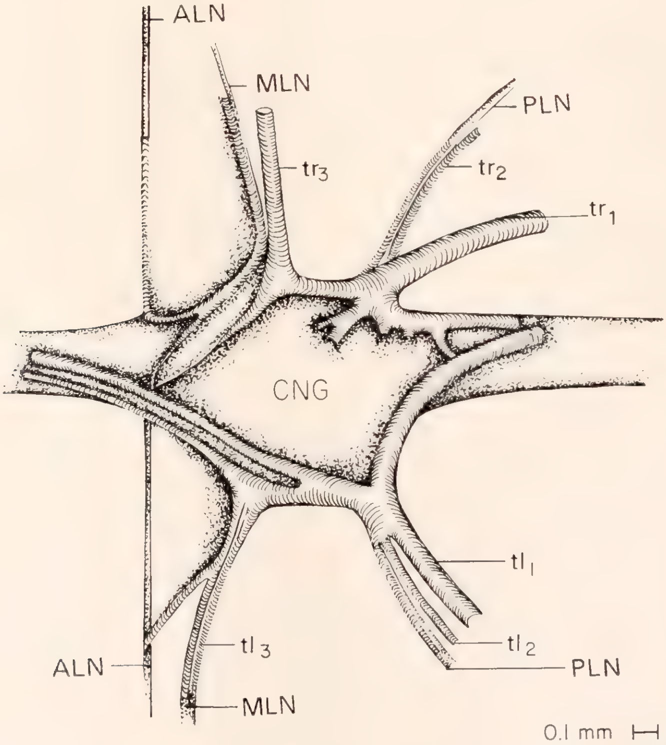

FIGURE 5. Gross anatomy of the immediate tracheal supply to the fourth abdominal ganglion; ALN—anterior lateral nerve; MLN—mid-lateral nerve; PLN—posterior lateral nerve; CNG—ganglion; tr_{1-3}, tl_{1-3}—tracheae.

At this point, the *intact* fourth abdominal ganglion of each of six pupae was perfused with its separate ganglionic flow, while the spiracles were perfused simultaneously with the spiracular flow, which consisted of the same or a different gas mixture. The perfusions were begun within a minute after the ganglion had been exposed and supported. The effects on spiracular valve behavior of surgery itself and of gas flow over the surface of the intact ganglion could be compared.

After these perfusions the delivery bell was raised, and tracheae tr_1, tr_2, tr_3, tl_1, tl_2, and tl_3 (Fig. 5) were cut at their junction with the ganglion; thus the ganglion was "detracheated." This permitted gases flowing over the ganglion to enter the open ends of tracheae attached to it, and evenutally to reach the cells in the ganglion. In addition those tracheal branches passing along and attached to the ventral nerve cord near the ganglion were punctured with a fine glass needle to avoid damaging the nerve cord, and then the tracheal ends were teased apart gently. To prevent fluid from creeping into the cut ends of the tracheae attached to the ganglion, thus blocking the ganglion from the experimental gas flow, the tracheae were not severed until just before the bell was lowered over the ganglion and the gas perfusions were begun. During intervals between experiments, air was perfused over the ganglion to prevent hypoxia.

To assess the long-term effects of detracheation, a plastic window could be sealed over the wound in the integument with melted paraffin. Pupae then were stored at room temperature as described above, and examined periodically.

Each experiment reported in this paper was performed on at least two and as many as eight pupae.

Departures from these general procedures as well as special methods will be explained in the appropriate sections below.

RESULTS

Effects of routine experimental procedures on spiracular valve behavior

Four major procedures—cannulation, use of spiracular windows, tracheal perfusion, and surgery—were followed routinely to prepare pupae for experiments. To evaluate the effects of each procedure, the normal behavior of the spiracular valves was used as the standard. Since valves of a pupa respiring in ambient air pulsate most of the time and occasionally flutter (Schneiderman, 1960), this pattern was chosen as "normal."

Cannulation. Four different diameters of polyethylene tubing were tested as cannulae. Spiracular valve responses to air perfusions through cannulae of different bores were the same. PE-10 tubing generally was used because it was the most convenient.

Five pupae were cannulated, each with a different combination of spiracles as cannulation sites. Spiracular valves of these pupae responded in the same way to air perfusions. Thus cannulation itself, the cannula bore size, and the sites of cannulation did not affect the behavior of spiracular valves.

Spiracular window. A plastic window was sealed over exposed valves to prevent the pupa from desiccating. The behavior of the spiracular valves of seven intubated pupae, first with and then without the window, was the same in response to air perfusions. Thus spiracular valve behavior was not visibly affected by the presence of a plastic window over the exposed spiracles.

Tracheal perfusion. The object of perfusing the tracheal system was to control the intratracheal gas composition. To meet this objective we considered the structure of the tracheal system and the rate at which gases were perfused through it. In addition we determined the time needed for equilibration of intratracheal gas with the perfused gas, the time needed for recovery from a perfusion, and whether valve responses to a given gas mixture are reproducible.

Gross dissection of a pupa reveals that tracheae form an interconnecting network. Large tracheae run longitudinally between spiracular manifolds from which numerous smaller transverse tracheae branch and rebranch repeatedly. There are no valves to cause unidirectional flow within pupal tracheae (Brockway and Schneiderman, 1967). Furthermore, when air is perfused through the tracheal system at a high rate, the pupa distends.

The flow rates used in these experiments were a compromise between rates high enough to insure constant average intratracheal levels of oxygen and carbon dioxide and rates low enough to prevent the pupa from distending. It was determined that a flow rate of 360 μl/min does not itself visibly affect valve behavior yet still meets the oxygen requirements of a pupa, whereas flow rates greater than 1430 μl/min cause the pupa to distend.

After 2 to 5 minutes of perfusion, valve behavior stabilized and usually remained the same until perfusion ended, usually 20 minutes but occasionally up to 4 hours later. In only eight of over 600 perfusions did valve behavior after 20 minutes differ from that after 2 to 5 minutes of perfusion. Moreover, results were highly reproducible from day to day in any individual and among individuals.

The resumption of normal valve behavior after perfusion had ended was interpreted as recovery and usually occurred within 5 to 10 minutes after perfusion ceases. The duration of perfusion had no visible effect on recovery time.

Similar valve responses to a given oxygen concentration occurred not only in the different spiracles of the same pupa but also among different pupae. Deviations were in the degree of response, not in different modes of behavior. For example, valves of 50 pupae fluttered at amplitudes ranging from low to high in response to perfusions of 15% O_2; there were no prolonged valve openings or valve constrictions in any pupa.

Surgery. Injury increases the metabolism of brainless diapausing pupae severalfold (Schneiderman and Williams, 1955). This increased metabolism is conspicuous about 24 hours after injury, becomes maximal about 4 days after injury, and persists from one to several weeks. It was important to know what effect surgery *per se* had on spiracular valve behavior, aside from any specific effect resulting from tracheal or neural surgery.

Each of 14 pupae that had cyclical respiratory activity before integumentary surgery was examined immediately after surgery for burst cycles. Only one pupa continued to exhibit cyclical respiratory activity after injury; its cycles were about 50 minutes long after surgery as compared to about 80 minutes before surgery. Valves of the 13 other pupae fluttered at low amplitude immediately after injury, although several minutes of constriction with pulsations were common.

The spiracular valve responses of injured pupae to different perfused gas mixtures were also examined, and compared to the responses of the same pupae to the same gas mixtures before injury. The valve responses of an individual pupa to a given perfused gas mixture were generally the same after injury as before. In five pupae, post-injury valve responses were recorded almost 72 hours after surgery, at a time when injury metabolism was near maximum. Changes in valve behavior after injury were limited to an increase in flutter amplitude, or to motionless, fully-open valves whereas before injury the fully-open valves occasionally closed partially.

Spiracular valve responses of intact intubated pupae to gas perfusions

It was necessary to know how the spiracular valves of intubated pupae that had not undegone surgery responded to various mixtures of carbon dioxide and oxygen. Thus pupae were perfused with gas mixtures in which the concentration of oxygen varied between 2 and 50%, and that of carbon dioxide ranged from 0 to 7%. Records of valve behavior under these conditions were obtained for each pupa, and served as a type of control record for subsequent experimental records for that same pupa. The following results were obtained:

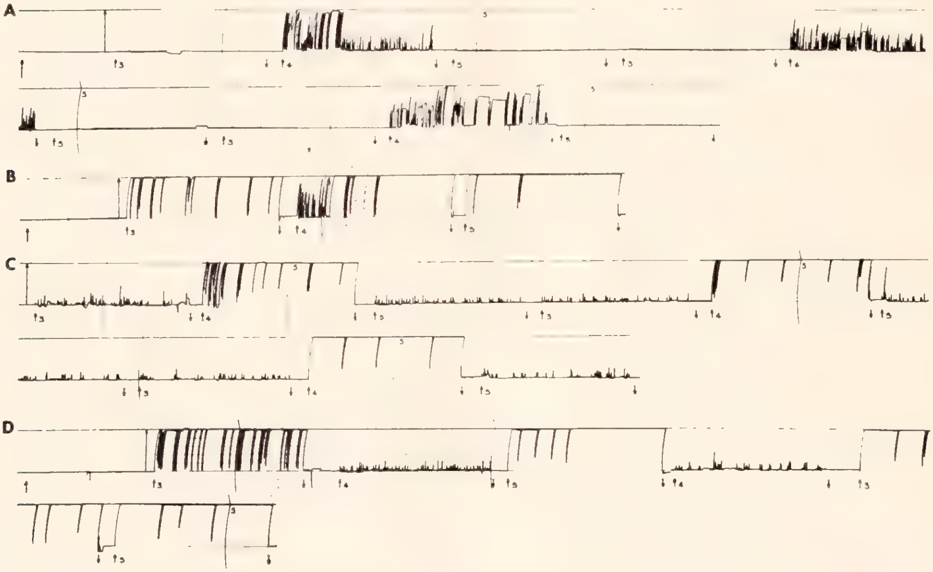

FIGURE 6. Spiracular valve responses to simultaneous spiracular and ganglionic perfusions following detracheation of the fourth abdominal ganglion. The large arrow at the beginning of each record indicates the amount that the pen was deflected when valves opened fully. The short arrows below the tracings indicate the beginning (upward-pointing arrows) and end (down-pointing arrows) of recording. The short breaks in recording represent the time required to bring the next valve in focus (chart speed = 0.5 mm/sec; each division on the chart represents 50 seconds); 3RAS, 4RAS, 5RAS—third, fourth, fifth right abdominal spiracle, respectively; (A.) Spiracular flow—50% O_2 + 3% CO_2; ganglionic flow—3% O_2 + 3% CO_2; (B.) Spiracular flow—3% O_2 + 3% CO_2; ganglionic flow—3% O_2 + 3% CO_2; (C.) Spiracular flow—21% O_2 + 3% CO_2; ganglionic flow—3% O_2 + 3% CO_2; (D.) Spiracular flow—3% O_2 + 3% CO_2; ganglionic flow—21% O_2 + 3% CO_2.

In 61 of 101 perfusions of 2 to 5% O_2 and 0 to 4% CO_2, valves opened; in the 50 other perfusions, valves fluttered at high amplitude. Since the concentration of carbon dioxide in all perfusions was too low to cause opening (Levy and Schneiderman, 1966a), the wide opening of the valves was a response to low oxygen.

When the concentration of carbon dioxide was 0 to 4%, while that of oxygen was 5 to 15%, valves fluttered in 143 of 151 perfusions.

When the concentration of carbon dioxide was 3% and oxygen was 50%, valves were constricted and motionless.

When the concentration of oxygen was too high to cause prolonged valve opening, *i.e.*, above 5%, valves could be induced to remain open by perfusion mixtures containing 7% or more carbon dioxide.

TABLE I

Effect of carbon dioxide concentrations on the valve behavior of the fourth right abdominal spiracle

Pupa no.	Gas mixture				Valve response* 4RAS**
	Spiracular		Ganglionic		
	% O_2	% CO_2	% O_2	% CO_2	
10–1			3	3	5
			5	3	2
			5	7	1
10–2			5	7	1
			8	12	1
			8	15	1
			8	20	1
			8	25	1
			8	30	1
			8	32	4
			8	35	6
			8	40	6
10–1	21	3	21	3	1
	21	3	5	7	1
	5	7	5	7	5
	5	7	21	3	5
	50	3	50	3	0
	50	3	5	7	0
	5	7	5	7	6
	5	7	50	3	6
10–2	8	3	8	3	1
	8	3	8	12	1
	8	12	8	12	5
	8	12	8	3	5

* 1 = closed, motionless; 2 = closed, pulsating; 3 = low amplitude flutter; 4 = medium amplitude flutter; 5 = fully open, moving; 6 = fully open, motionless.

** 4RAS = fourth right abdominal spiracle.

In summary, intratracheal carbon dioxide concentrations of 7% or more were required to cause valves to open fully and remain open when the intratracheal oxygen concentration was 5 to 21%. When the carbon dioxide concentrations were too low to cause valve opening, the valves behaved as follows: they opened fully or fluttered at high amplitude in 2 to 5% oxygen; fluttered at low amplitude in 5 to 15% oxygen; constricted in 21% oxygen or higher. These results agree well with those obtained earlier by other methods (Schneiderman, 1960; Levy and Schneiderman, 1966a, 1966b).

TABLE II

Effect of oxygen concentrations on the valve behavior of the fourth right abdominal spiracle

Pupa no.	Gas mixture				Valve response* 4RAS**
	Spiracular		Ganglionic		
	% O_2	% CO_2	% O_2	% CO_2	
10–1	3	3	3	3	5
	3	3	21	3	1
	21	3	21	3	1
	21	3	3	3	3
	5	3	5	3	2
	5	3	21	3	1
	21	3	21	3	1
	21	3	5	3	2
10–2	3	3	3	3	5
	3	3	21	3	1
	21	3	21	3	1
	21	3	3	3	5

* 1 = closed, motionless; 2 = closed, pulsating; 3 = low amplitude flutter; 4 = medium amplitude flutter; 5 = fully open, moving; 6 = fully open, motionless.

** 4RAS = fourth right abdominal spiracle.

Control of spiracular valve behavior

To determine whether the central nervous system responds to intratracheal oxygen or carbon dioxide, or to oxygen or carbon dioxide dissolved in the body fluids, the exposed fourth abdominal ganglion (with its tracheae intact) was perfused about 6 hours after the square of integument had been removed. When both the ganglion and the tracheal system were perfused simultaneously with the same gas mixture, spiracles three, four, and five responded similarly. Moreover, all valves behaved alike even when the ganglion and the tracheal system were

TABLE III

Effect of oxygen concentration on the CO_2-trigger threshold for spiracular valve opening

Pupa no.	Gas mixture				Valve response*		
	Spiracular		Ganglionic				
	% O_2	% CO_2	% O_2	% CO_2	4RAS	3RAS	5RAS**
10–1	8	7	21	3	1	3	3
	5	7	21	3	5	5	5
	5	7	50	3	6	6	6
10–2	5	7	21	3	6	6	6

* 1 = closed, motionless; 2 = closed, pulsating; 3 = low amplitude flutter; 4 = medium amplitude flutter; 5 = fully open, moving; 6 = fully open, motionless.

** 3RAS = third right abdominal spiracle; 4RAS = fourth right abdominal spiracle; 5RAS = fifth right abdominal spiracle.

perfused simultaneously, each with a different gas mixture. Apparently tracheae are not very permeable to externally applied gases. It is the tracheal gas *per se* that affects the ganglion, not gases dissolved in the hemolymph (or Kel-F oil) surrounding the ganglion.

The key steps in these experiments were detracheating and perfusing the ganglion with ganglionic flows. Tables I–III include representative data taken from recordings of valve responses to various perfusions following detracheation. Figure 6 shows sample records. Table I shows the effects of carbon dioxide in the ganglionic flow on the valve behavior of the fourth spiracle. When the carbon dioxide concentration was 7 to 30%—concentrations that have been demonstrated repeatedly to cause valve opening when the spiracles are perfused with them—the spiracle remained constricted and pulsating. The carbon dioxide concentration over the ganglion had to be above 30% (pupa 10–2) before the fourth spiracle could be induced to open. Physiological concentrations of carbon dioxide (3 to 7%) perfused through the ganglion had no effect on spiracular behavior. When the spiracles were perfused with 5% O_2 + 7% CO_2, all the spiracular valves opened fully although the ganglionic flow was 50% O_2 + 3% CO_2. On the other hand, 5% O_2 + 7% CO_2 perfused over the ganglion did not cause valve opening of the fourth spiracle. Similarly, a ganglionic flow of 8% O_2 + 12% CO_2 failed to evoke valve opening by the fourth spiracle, although all valves opened fully when the spiracles were perfused with the same mixture.

These data show that the behavior of the spiracles is not affected when the carbon dioxide concentration of the ganglionic flow is in the physiological range, *i.e.*, there is no central carbon dioxide effect. The primary target of carbon dioxide is outside the central nervous system, *i.e.*, peripheral.

The results of 30 of 33 simultaneous spiracular and ganglionic perfusions indicate that the ganglion responded to its own concentration of oxygen, not to that of the spiracular flow (Table II). When 21% O_2 + 3% CO_2 was perfused through the ganglion, the valve of the fourth spiracle closed although the spiracular flow contained 3% O_2 + 3% CO_2 (pupa 10–2). The valve of the fourth spiracle of pupa 10–1 could be induced to flutter by a ganglionic flow of 5% O_2 + 3% CO_2; the valve could be closed again by a ganglionic flow of 21% O_2 + 3% CO_2.

These data show that the ganglion responds to changes in the oxygen concentration of its own gas supply, *i.e.*, there is a central oxygen effect. The data also indicate that each ganglion serves its own spiracles.

Table III shows the effect of the concentration of oxygen in the ganglionic flow on the CO_2-trigger threshold for spiracular valve opening. As shown earlier, a spiracular flow of 5% O_2 + 7% CO_2 causes full opening of all valves. The fourth spiracular valve of pupa 10–1 continued to open fully in 5% O_2 + 7% CO_2 even when the concentration of oxygen in the ganglionic flow was increased to 50%. The few available data indicate that the concentration of oxygen in the ganglionic flow apparently has no effect on the CO_2-trigger threshold for valve opening.

Table III also shows one example of the effect of the spiracular oxygen concentration on the CO_2-trigger threshold for spiracular valve opening. When carbon dioxide was kept constant at 7% and the spiracular oxygen level increased from 5 to 8%, valve responses of the control spiracles changed from fully open in 5% O_2 to flutter in 8% O_2. The fourth spiracular valve, which had been fully open in 5% O_2 + 7% CO_2, constricted and pulsated when the spiracular flow was 8% O_2 + 7% CO_2, and thus responded differently from its control spiracles.

This experiment demonstrated that the spiracular, not the ganglionic, oxygen concentration affects the CO_2-trigger threshold for valve opening. It also provided additional evidence that each ganglion serves its own spiracles.

However, further experiments were performed to locate the primary site of nervous control of spiracular activity. The ventral nerve cord was cut either posterior to, anterior to, or posterior and anterior to the fourth abdominal ganglion, thus isolating the ganglion from nerve impulses arising in ganglia either posterior and or anterior to it. Table IV contains data derived from recordings of valve responses of pupae that had undergone nerve cord transection. When 50% O_2 + 3% CO_2 was perfused through the ganglion, the fourth spiracle fluttered (pupa 10–2); its controls oscillated about the fully open position in response to a spiracular perfusion of 3% O_2 + 3% CO_2. In most perfusions the behavior of the fourth spiracle differed from that of its controls. Cutting the nerve cord and isolating the fourth abdominal ganglion did not appear to affect the behavior of the fourth spiracle which responded to the ganglionic flow. These results confirm other evidence that a spiracle is served primarily by its own ganglion, not by the ganglion in an adjacent segment.

To further elucidate the role of the nervous system in controlling spiracular behavior, the spiracular closer muscle was partially denervated by cutting the

TABLE IV

Effect of nerve cord transection on valve behavior

Pupa no.	Position of cut	Gas mixture				Valve response*					
		Spiracular		Ganglionic		Before transection			After transection		
		% O_2	% CO_2	% O_2	% CO_2	4RAS	3RAS	5RAS	4RAS	3RAS	5RAS**
7–1	Anterior to 4AG	10	3	21	0	2	4	4	6	3	3
		21	0	10	3	4	2	2	2	2	2
7–2	Anterior to 4AG	10	3	21	0	2	4	4	2	2	2
		21	0	10	3	3	2	2	4	2	2
7–3	Posterior to 4AG	10	3	21	0	2	4	4	2	4	4
		21	0	10	3	2	4	4	4	2	2
7–4	Anterior and posterior to 4AG	10	3	21	0	2	4	4	2	4	4
10–2	Anterior to 4AG	5	3	3	3	5	2	2	0	0	0
		3	3	50	3	6	6	6	4	5	5

* 1 = closed, motionless; 2 = closed, pulsating; 3 = low amplitude flutter; 4 = medium amplitude flutter; 5 = fully open, moving; 6 = fully open, motionless.

** 3RAS = third right abdominal spiracle; 4RAS = fourth right abdominal spiracle; 5RAS = fifth right abdominal spiracle.

mid-lateral nerve (Fig. 3) where it leaves the ganglion. The integumentary wound was sealed with a plastic window. Spiracular gas perfusions and recordings of the valve behavior of the fourth right abdominal spiracle and its contralateral and adjacent control spiracles were begun about 9 minutes later. Perfusions and records of valve behavior were made at various times up to 30 days after nerve transection. The partially denervated spiracle remained open for 24 hours even in 50% O_2, while its controls fluttered in 21% O_2 and closed in 50% O_2. Beginning at about 72 hours and continuing as long as 20 days after denervation, the fourth spiracle closed in response to spiracular perfusions of 21 and 50% O_2, but opened fully in 10% O_2. Thereafter the fourth spiracle remained closed but could be induced to open by perfusions of 100% CO_2 for about one minute; the controls continued to flutter in 21 and 10% O_2, and to constrict in 50% O_2.

These results show that the partially denervated spiracular muscle, like the completely denervated spiracular muscle, is capable of responding to carbon dioxide and oxygen (Beckel and Schneiderman, 1957). However, partial denervation alters the degree of the muscle's responses to changes in the oxygen concentration. In addition the muscle's hypersensitivity to a concentration of oxygen that caused its normal controls to flutter was demonstrated.

Discussion

Several procedures used in these investigations are either described for the first time or are modifications of techniques reported in other papers. Since interpreting the results depends on understanding the advantages and limitations of each method used, two key experimental approaches, intratracheal perfusion and detracheation of the ganglion, are discussed before the results are interpreted.

It is important not only to know the tracheal gas composition but also to be able to control it. One way of doing this is opening the pupal tracheal system to ambient gases of known composition by inserting short tubes past the spiracular valves (Buck and Keister, 1955; Schneiderman, 1960). This approach has the advantage of not affecting the animal by mass internal flows. The tracheal gas composition is not known with certainty "but at least it can be kept constant and the oxygen and carbon dioxide tensions can be varied independently" (Schneiderman, 1960, page 520). The behavior of spiracles when the pupal tracheal system was perfused with various gases was very similar to the behavior of spiracles when the insect was placed in the same gases. However, intratracheal perfusion makes it possible to control the average tracheal gas composition.

Detracheating a ganglion cuts its normal oxygen supply route, thus making the ganglion hypoxic. One would expect the valves of the same segment to gape as they do initially after exposing a denervated spiracular muscle to low oxygen (Beckel and Schneiderman, 1957), and this happened. Detracheating a ganglion produces "functional denervation" of the pair of spiracular muscles it serves. The fact that the detracheated ganglion perfused with appropriate gas mixtures did not cause the spiracles to gape is evidence that the method of perfusing the ganglion worked.

The interaction between carbon dioxide and oxygen in triggering bursts in diapausing pupae of Lepidoptera has been described (Schneiderman, 1960; Levy and Schneiderman, 1966b). Their results, based both on observations of spiracular valve behavior and on analyses of tracheal gases, showed that the triggering carbon dioxide concentration varied with the tracheal oxygen concentration. The carbon dioxide concentration needed to cause prolonged valve opening increased as the intratracheal oxygen concentration increased. Results of the present experiments confirm these earlier reports. Moreover, our data also illuminate another aspect of oxygen-carbon dioxide interaction, the effect of the carbon dioxide concentra-

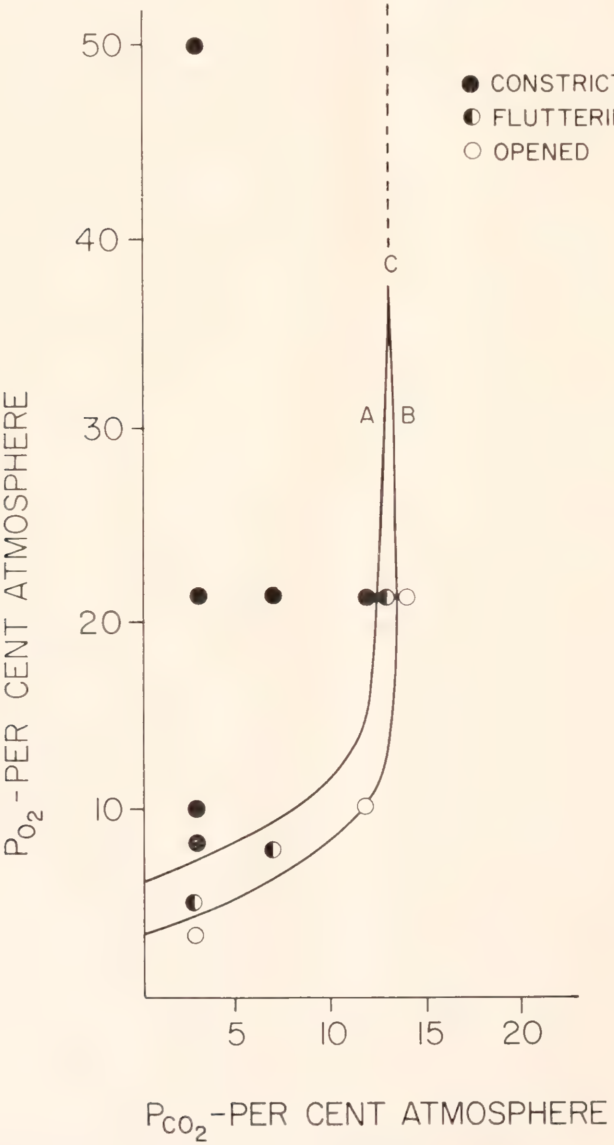

FIGURE 7. Oxygen-carbon dioxide interaction curves for pupa 10–2. See text for details.

tion on the O_2-flutter threshold of the valves (the concentrations of oxygen at which fluttering begins).

If one plots the intratracheal oxygen-carbon dioxide concentrations at which flutter occurs, in addition to the concentrations of these gases that cause valve opening, the two resulting curves define valve behavior over a range of oxygen and carbon dioxide concentrations. Using data obtained from perfusion experiments, such dual O_2-CO_2 interaction curves were constructed (Fig. 7). The area between curves A and B includes all combinations at which flutter was observed; the area between the ordinate and curve A includes all combinations at which pulsation or constriction occurred. The area delimited by curve B, the ordinate, and the abscissa includes all combinations for which valves opened. These curves show that when the level of carbon dioxide is sufficiently high, flutter occurs at oxygen concentrations to which valves usually respond by constricting. Thus flutter occurred in 21% O_2 when the carbon dioxide concentration was about 14%; the valves of this same pupa constricted and pulsated in 21% O_2 when the carbon dioxide concentration was 3%. Levy and Schneiderman (1966a) acknowledged the possibility that carbon dioxide could influence the O_2-flutter threshold but they stated that "until P_{CO_2} rises above 6% it exerts no marked influence on the response of the spiracles to P_{O_2}" (page 97). Results of the present experi- (In the normal respiratory activity of diapausing Cecropia pupae, flutter can and whereas marked effects are evident at carbon dioxide concentrations above 10%. ments confirm their conclusion, and reveal no conspicuous effects of carbon dioxide on the O_2-flutter threshold when the carbon dioxide concentration is below 7%, does occur when the intratracheal oxygen concentration is between 18 and 21%. This phenomenon will be discussed in a subsequent paper (Burkett and Schneiderman, 1974).)

Further evidence that increasing the concentration of carbon dioxide affects the flutter threshold comes from re-examining data derived from tracheal gas analyses (Levy and Schneiderman, 1966b, pages 108, 109, Figures 2a, 3a). If these data are equated to corresponding valve behavior, they can be plotted to show the same relationships as in Figure 7, confirming qualitatively the interrelationships of carbon dioxide and oxygen on the flutter threshold.

The following experimental results indicate that in the diapausing Cecropia pupa, carbon dioxide affects spiracular behavior by acting peripherally on the spiracular mechanism and not on the central nervous system. The fourth abdominal spiracle did not respond when the fourth abdominal ganglion was perfused with pure carbon dioxide. Neither the experimental spiracle nor its controls opened fully and remained motionless when the *detracheated* fourth abdominal ganglion was perfused with gases in which the carbon dioxide concentration ranged from 7 to 30%. The same gas mixtures perfused systemically caused full and prolonged opening of all valves. Hence in the normal respiratory cycle, as intratracheal carbon dioxide increases, the spiracular mechanism responds long before tracheal carbon dioxide gets high enough to affect the central nervous system.

There is no evidence from these experiments that carbon dioxide concentrations below 30% affect spiracular behavior either directly or indirectly via the central nervous system. The possibility remains that a direct or indirect peripheral response to carbon dioxide may be transmitted over sensory fibers to the central nervous system, which then alters the flow of information to the spiracular muscle. No specific receptors that may respond to changes in the concentration of carbon dioxide or oxygen have been found in the spiracular region although Beckel (1958) made an extensive histological search for such sensory elements. That the spiracular muscle can behave autonomously was shown by studies on the denervated muscle, which relaxed in high concentrations of carbon dioxide (Schneiderman, 1956; Beckel and Schneiderman, 1957); present results of partial denervation studies confirm their observations.

Hoyle (1960) concluded that in the locust spiracular muscle the partial pressure of carbon dioxide in the tracheae near the spiracle triggers spiracular opening. The present study indicates that this is also true for Cecropia pupae.

Van der Kloot (1963) passed 10% CO_2 + 90% O_2 over the isolated spiracular nerve-muscle preparation of a Cecropia pupa; he found that firing in the anterior lateral nerve ceased but some firing continued in the mid-lateral nerve. This indicates a central effect of carbon dioxide on nerves connected with the spiracular muscle. In contrast, our studies indicate that in the intact pupa carbon dioxide acts directly on the spiracular mechanism, not on the central nervous system. However, the possibility exists that sensory information concerning the carbon dioxide concentration or degree of stretch in the spiracular muscle is transmitted to the central nervous system, and this sensory input may affect motor output to the muscle.

Oxygen appears to act by a separate mechanism. In the present experiments the valve behavior of the fourth spiracle could be "dictated" by perfusing the detracheated ganglion with a gas of an appropriate concentration of oxygen. Hence oxygen appears to act via the central nervous system. The evidence for a peripheral response to oxygen is inconclusive but it appears that spiracular perfusions in which the oxygen concentration is 21% or more can mask a central response to oxygen. When the concentration of oxygen in the spiracular perfusion was above 21%, the fourth spiracle occasionally ceased fluttering even though the ganglionic oxygen concentrations used (5 to 10%) usually induced fluttering. This suggests some peripheral effect of oxygen at high concentrations. But in the normal physiological range of oxygen concentrations (5 to 21%) we have never observed a peripheral oxygen effect.

With regard to the nervous control of spiracular valve behavior, the results of the present studies are best evaluated by first considering the results of earlier studies (Beckel, 1958; Van der Kloot, 1963). Electrophysiological studies on the spiracular nerve and closer muscle (Van der Kloot, 1963) indicated that nerve A8 which innervates the muscle (Beckel, 1958) is made up of at least four axons, of which two are from the mid-lateral nerve (Fig. 3). When the anterior lateral nerve was stimulated, the muscle twitched, and Van der Kloot concluded that in the intact pupa motor output from the anterior lateral nerve stimulates the closer muscle to contract. He also noted that when the carbon dioxide concentration was increased, efferent input through anterior lateral nerve ceased. This observation has important implications, for Beckel (1958) stated that the anterior lateral nerve is the median nerve which "arises in the ganglion preceding and courses between the ensheathed connectives to arise from the ganglion succeeding" (page 91). When the mid-lateral nerve was stimulated, there was a delay before an action potential was observed in the anterior lateral nerve (Van der Kloot, 1963), and Van der Kloot concluded that fibers of the two synapse. Furthermore, nerve cord transection between the third thoracic and first abdominal ganglia eliminated the action potential in the anterior lateral nerve that resulted from stimulating the mid-lateral nerve. This result indicated that the synapse lay outside the ganglion of the stimulated mid-lateral nerve, and that the central nervous control of a spiracle lies in the ganglion of a different segment from that of the spiracle.

Van der Kloot found that the contracting spiracular muscle also receives impulses from the mid-lateral nerve, although the role of these axons is not known. In addition he found that even when motor output over the anterior lateral nerve to the muscle ceased (as it did when carbon dioxide was blown over the preparation), the muscle did not always relax since the muscle itself generated potentials. He drew no conclusions as to the exact roles of either nerve in the intact pupa but he suggested two possible functions for the spiracular nerve: The innervated muscle depends in part on excitatory output from the CNS to contract, and the CNS sends inhibitory impulses to the muscle when the carbon dioxide concentration increases.

The following results of the present experiments provide an alternative picture, and support the view that the ganglion and the spiracles which it primarily controls are located in the same segment and that the mid-lateral nerve plays a major role in triggering contraction of the spiracular muscle: (1) Variations in the local oxygen concentration to the fourth abdominal ganglion affect the fourth abdominal spiracle. (2) Detracheating the ganglion has the same effect as denervating the spiracle but does not affect adjacent spiracles. (3) Cutting the nerve cord between the third and fourth, and between the fourth and fifth abdominal ganglia does not change the responses of the fourth spiracle to spiracular or ganglionic perfusions of various gas mixtures. Nor does this operation affect the responses of the third and fifth spiracles although each was deprived of innervation from the fourth ganglion. (4) The behavior of the two spiracles in a segment is coordinated with each other but is not strictly synchronous with that of spiracles in other segments (Van der Kloot, 1963; Brockway and Schneiderman, 1967).

Cecropia pupae exhibit discontinuous respiration during which oxygen uptake is continuous whereas carbon dioxide release occurs periodically in "bursts." A carbon dioxide burst begins whenever the intratracheal concentration of carbon dioxide reaches the triggering threshold. The emphasis in this paper on the regulation of spiracular behavior by carbon dioxide and oxygen should not obscure the fact that the adaptive significance of discontinuous respiration, whether in insects or in other terrestrial arthropods (Robinson and Paim, 1969) is water conservation (Buck, 1958). What happens to the Cecropia pupa when the temperature in nature falls too low for the neuromuscular activity associated with discontinuous respiration to continue is discussed in a subsequent paper (Burkett and Schneiderman, 1974).

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SUMMARY

1. A technique was developed for perfusing the entire tracheal system of an insect with a known gas mixture at a rate that insured constant average intratracheal concentrations of oxygen and carbon dioxide. In addition to this technique, a method was devised to provide a ganglion with its own gas supply, separate from that of the rest of the insect. These techniques enabled us to show the following:

2. The behavior of the spiracles is unaffected when physiological concentrations of carbon dioxide are perfused through the ganglion. However, carbon dioxide perfused through the spiracles does affect valve behavior. Hence the primary response to carbon dioxide is peripheral.

3. The behavior of the spiracles is affected by varying the concentration of oxygen through the ganglion. Hence the primary response to oxygen is central.

4. Central nervous control of spiracular behavior resides primarily in the ganglion of the same segment in which spiracles are located.

5. Not only does oxygen affect the CO_2-trigger threshold but also carbon dioxide affects the O_2-flutter threshold. Moreover, the interaction between oxygen and carbon dioxide occurs peripherally.

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DISCONTINUOUS RESPIRATION IN INSECTS AT LOW TEMPERATURES: INTRATRACHEAL PRESSURE CHANGES AND SPIRACULAR VALVE BEHAVIOR

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Insects face the problem of conserving body water. This problem is especially acute for pupae of the wild silkworm *Hyalophora cecropia* which in the northern parts of their range overwinter in environments where the temperature may drop to —30° C and below. At these low temperatures the humidity is also low, and there is a tendency for the animals to desiccate. Yet this rarely happens although pupae neither feed nor drink. Their only source of water, in addition to the body water with which they enter the pupal stage, is metabolic water.

Most pupal water loss occurs through the spiracles. When the spiracles open, air enters the tracheae and carbon dioxide and water vapor leave. Any mechanism that keeps the spiracles closed will enable the insect to retain water. Buck (1958) suggested that discontinuous respiration is such a mechanism.

Discontinuous respiration occurs cyclically; each cycle consists of a burst, a constriction period, and a flutter period which is followed by another burst, thus marking the beginning of another cycle. During the burst spiracular valves remain open for several minutes and then close for some time in the constriction period. Following the constriction period, valves begin opening and closing continuously or "fluttering," and this is the flutter period. Discontinuous respiration in Cecropia pupae is well-documented but evidence that these pupae exhibit discontinuous respiration at the low temperatures they experience is conflicting. Levy and Schneiderman (1966) reported cycles at 8.5° C, and Brockway (1964) recorded cycles at 0° C. However, Kanwisher (1966) concluded that there is no discontinuous respiration below 10° C and that pore diffusion accounts for gas exchange at low temperatures.

Understanding how these pupae conserve water at low temperatures and low humidities depends mostly on understanding how the spiracles behave under such conditions. This report examines the effects of low temperature on cyclical respiratory activity and on the behavior of spiracular valves.

MATERIALS AND METHODS

Experimental animals

Diapausing pupae of *Hyalophora cecropia* had their brains removed three months before the experiment began to insure permanent diapause (Williams, 1946). Pupae then were stored at 22–25° C and 80–90% R.H. until used.

To record intratracheal pressure changes, the spiracles of pupae were cannulated as described previously (Burkett and Schneiderman, 1974). The pupae then were placed in a water-saturated environment for two weeks to recover.

Recording intratracheal pressure changes

Intratracheal pressure changes were recorded continuously except during temperature equilibrations. The methods used were similar to those described in previous papers (Schneiderman and Schechter, 1966; Brockway and Schneiderman, 1967). Pressure changes as small as 0.025 mm Hg could be detected (Brockway and Schneiderman, 1967). As in these earlier papers it should be emphasized that the transducer repsonded to pressure changes in a system composed of the tracheal system, the cannula, and the transducer itself. Hence the recorded pressure changes were smaller than the actual intratracheal pressure changes that occurred in an intact pupa.

Recording spiracular valve behavior

Beckel (1958) described the anatomy of the spiracles of Cecropia in detail. The width of the spiracular opening is regulated by the spiracular valve which is moved by a closer muscle.

In one set of experiments reported here spiracular valve movements and intratracheal pressure changes were recorded simultaneously. To observe the valves of the third, fourth, and fifth right abdominal spiracles, they were exposed by scraping away the filter apparatus and gently pushing aside the peritreme with a hot needle. To keep the valves moist, a single transparent plastic window was sealed in place over them with melted paraffin. The valves were observed daily to insure that no sticking or desiccation had occurred.

The method described previously (Burkett and Schneiderman, 1974) for recording valve movements was used. Valve movements throughout at least one complete respiratory cycle were recorded at 20°, 15°, and 10° C. Below 10° C very long cycles precluded continuous direct observations and recordings of valve movements; thus 20-minute records with 20-minute intervals between recordings were made during the flutter and constriction periods of the cycle, and usually throughout the entire burst period. At − 10° to − 20° C valves were observed for 20-minute periods once every two hours.

Recording of intratracheal pressure changes at different temperatures

Intratracheal pressure changes of two pupae were studied by immersing the pupa-transducer system in a water-ethylene glycol bath. This method worked well at temperatures above 0° C. But at 0° C and below, recording of intratracheal pressure is affected noticeably by changes in room temperature and pressure. For this reason, and to permit observation of the spiracular valves at low temperatures, a different approach was developed. A pupa and the pressure transducer to which it was attached were placed in a small deep freezer fitted with a viewing window (Fig. 1).

The pupa and transducer were equilibrated at each temperature for 24 hours before recording was begun. Temperatures were lowered successively from + 25°

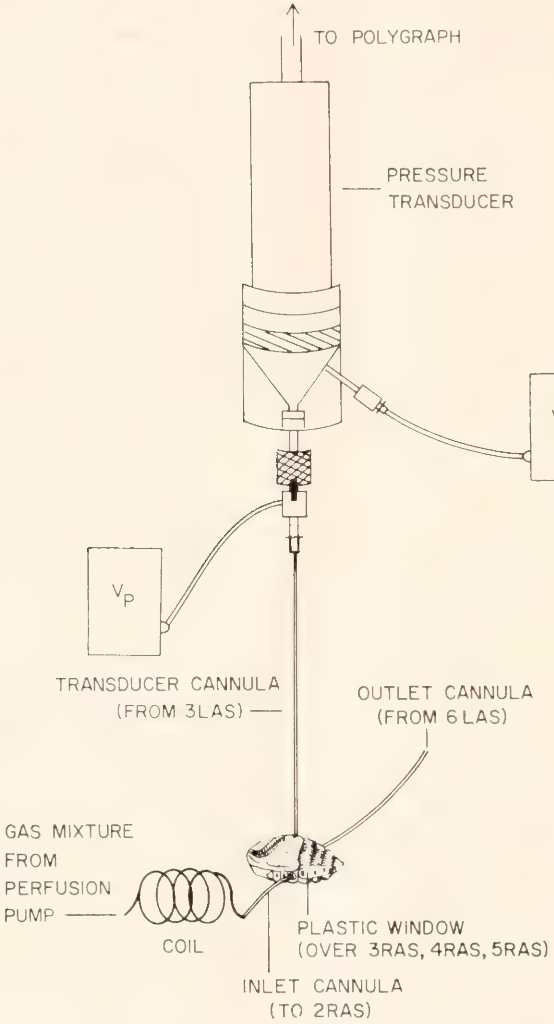
TO POLYGRAPH
PRESSURE TRANSDUCER
V_T
V_P
TRANSDUCER CANNULA (FROM 3LAS)
OUTLET CANNULA (FROM 6LAS)
GAS MIXTURE FROM PERFUSION PUMP
COIL
PLASTIC WINDOW (OVER 3RAS, 4RAS, 5RAS)
INLET CANNULA (TO 2RAS)

FIGURE 1. This assembly for simultaneously recording intratracheal pressure and valve movements was placed in a freezer, the temperature of which could be held constant to within ± 0.5° C at each experimental temperature between ambient and − 20° C. An infusion pump forced the experimental gas, cooled in the coil to the temperature of the freezer, into the tracheal system via the "inlet" cannula in the second right abdominal spiracle (2RAS). The "outlet" cannula in the sixth left abdominal spiracle (6LAS) prevented pressure build-up as a result of intratracheal perfusion. The cannula in the third left abdominal spiracle (3LAS) connected the tracheal system to the pressure transducer. To establish the baseline of intratracheal pressure records when the temperature was changed, and to check the size of a developing intratracheal vacuum without opening the freezer, the tracheal system was opened to the atmosphere by using two-way normally closed solenoid control valves (Allied Control Company, Plantsville, Connecticut). When the control valve of the transducer (V_t) was energized, fluid would have been forced from the transducer dome into the pupa had a second valve (V_p) not been used to equilibrate pressure. Movements of the third, fourth, and fifth right spiracular valves (3RAS, 4RAS, 5RAS) were observed and recorded as described earlier (Burkett and Schneiderman, 1974).

or + 20° C to — 20° C in 5° intervals, and they were increased similarly to + 20° C after recording at − 20° C.

Intratracheal perfusions at low temperatures

In one series of experiments the responses of the spiracular valves to oxygen and carbon dioxide at low temperatures were examined. The composition of the intratracheal gas was regulated by perfusing known concentrations of oxygen and carbon dioxide through the tracheal system as described earlier (Burkett and Schneiderman, 1974).

Table I

Summary of data from records of intratracheal pressure and valve movements of pupa II at different temperatures

Temperature (°C)	No. of complete cycles recorded	Average length of cycle (min)	Average length of burst (min)	"Open" phase (min)	"Decline" phase (min)	Average length of constriction (min)	Average intratracheal vacuum developed during constriction (mm Hg)	Average length of flutter (min)	Average number of valve movements/min
+20	6	341	30 (8.8)*	21 (70)	9 (30)	41 (12.0)	−1.99	270 (79.2)	32
+15	6	365	72 (19.8)	59 (82)	13 (18)	56 (15.4)	−2.35	237 (64.8)	25
+10	3	706	85 (12.0)	45 (53)	40 (47)	104 (14.7)	−4.5	517 (71.8)	19
+5	6	839	85 (10)	34 (40)	51 (60)	377 (45)	−5.24	377 (45)	10
0	1(II) 2(III)	1517†	180† (11.9)	80† (44.4)	100† (55.6)	187(II) (12.3)	−3.13(II)	1229† (80.9)	1
−5	0	>16,200	Data incomplete	—	—	—	—	16,180	1/min-1/hr
−10	0	—	—	—	—	—	—	—	0
−15	0	—	—	—	—	—	—	—	0
−20	0	—	—	—	—	—	—	—	0

* Numbers in parentheses indicate per cent of cycle occupied by a given phase. Those for open and decline phases of burst are per cent of burst.

† Average of all such phases recorded from two pupae (II and III).

The experimental gas mixture was cooled to the temperature of the freezer before the mixture entered the tracheal system. Ten minutes were allowed for complete tracheal perfusion and equilibration with the gas before valve responses to the mixture were recorded. At least two hours were allowed for recovery from one gas mixture before a different mixture was introduced into the tracheal system. Air was perfused through the tracheal system during the recovery period.

Other procedures will be discussed in the appropriate sections below.

At least three cycles were recorded for each pupa at 5° intervals from + 20° to + 5° C. To record three complete cycles at 0° C two pupae wre used. No complete cycles were recorded below 0° C.

RESULTS

Effects of decreasing temperatures on cyclical respiratory activity

Tables I, II, and III summarize data obtained from records of intratracheal pressure changes in one pupa. These data are representative of those obtained from all pupae used in these experiments. Figures 2, 3, and 4 show portions of the respiratory cycle at 0° and − 5° C.

The following changes in cyclical respiratory activity occurred as the ambient temperature decreased: (1) Discontinuous respiration persisted down to − 5° but not at − 10° C or below. (2) The duration of the respiratory cycle increased as the temperature decreased. Cycle length approximately doubled between 20° and 10° C, and between 10° and 0° C. (3) At temperatures above 0° C the pattern of the respiratory cycle was unchanged, that is, cycles were made up of a burst, during which valves remained fully open; a constriction period, during which valves remained closed; a pressure rise period following constriction, during which valves opened fully for several seconds and then closed again; and a flutter period, during which valves continuously opened briefly and then closed for several seconds. At 0° C, however, bursts and flutters persisted but constriction disappeared in some cycles. At − 5° C constriction disappeared altogther.

TABLE II

Average length (seconds ± s. e.) of microcycles during different parts of the flutter period

Temperature (°C)	Part of flutter period		
	Beginning	Middle	End
20	57.4* ± 41.03** (10–160)†	58.53 ± 32.71 (20–134)	43.2 ± 16.24 (10–70)
15	54.83 ± 8.03 (10–120)	67.00 ± 27.07 (20–230)	47.4 ± 16.92 (10–80)
10	68.30 ± 47.84 (20–170)	76.33 ± 52.83 (20–200)	42.40 ± 24.77 (10–85)
5	202.97 (4–833)	229.73 ± 223.19 (20–820)	125.77 ± 109.39 (10–200)
0	452.43 ± 418.12 (20–1040)	809.57 (20–6864)	497.83 (30–3160)
−5	460 ± 423.72†† (60–7330)	—	—

* Each average figure is based on a total of 30 microcycles (ten from each of three cycles) from records of one pupa.

** S. D.

† Range.

†† Based on all microcycles recorded.

Changes in burst, constriction, and flutter

The percentage of a total cycle occupied by the burst did not change systematically as temperature decreased (Table I). The burst itself is made up of two distinct phases (Schneiderman, 1960): an "open" phase, during which

TABLE III

Average intratracheal vacuum (mm Hg ± s. e.) developed during microcycles in different parts of the flutter period

Temperature (°C)	Part of flutter period		
	Beginning	Middle	End
20	−0.113* ± 0.067** (−0.025 to −0.125)†	−0.096 ± 0.047 (−0.025 to −0.125)	−0.068 ± 0.041 (−0.025 to −0.050)
15	−0.089 ± 0.071 (−0.025 to −0.125)	−0.089 ± 0.045 (−0.025 to −0.150)	−0.080 (−0.025 to −0.050)
10	−0.188 ± 0.180 (−0.025 to −0.200)	−0.105 ± 0.075 (−0.025 to −0.250)	−0.067 ± 0.041 (−0.025 to −0.125)
5	−0.256 (−0.075 to −0.300)	−0.204 (−0.150 to −0.250)	−0.060 ± 0.037 (−0.025 to −0.150)
0	−0.260 ± 0.182 (−0.025 to −0.200)	−0.353 (−0.150 to −0.375)	−0.073 ± 0.049 (−0.025 to −0.150)
−5	−0.149†† (−0.04 to −2.273)		

* Each average figure is based on a total of 30 microcycles (ten from each of 3 cycles) from records of one pupa.

** S. D.

† Figures in parentheses indicate ranges of microcycles examined.

†† Based on all microcycles recorded.

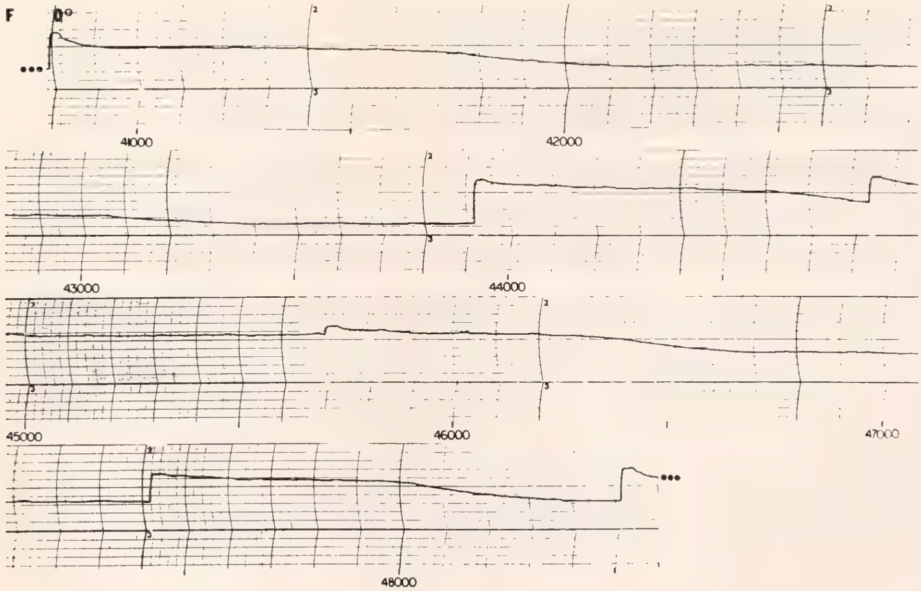

FIGURE 2. Intratracheal pressure record of pupa II at 0° C. Since this particular cycle lasted 28 hours, only a portion of the flutter period immediately preceding the burst is shown. The chart speed in this and subsequent records was 0.25 mm/sec unless otherwise indicated. Thus one small division between two thin vertical lines on the chart represents 20 seconds, and one large division between two heavy vertical lines represents 100 seconds. Recordings in this and other intratracheal pressure records were made at a sensitivity of 0.01 mV/cm.

valves are fully open and generally motionless; and a "decline" phase, during which valves oscillate about the fully open position. The percentage of the burst occupied by the decline phase increased as the temperature dropped below 10° C;

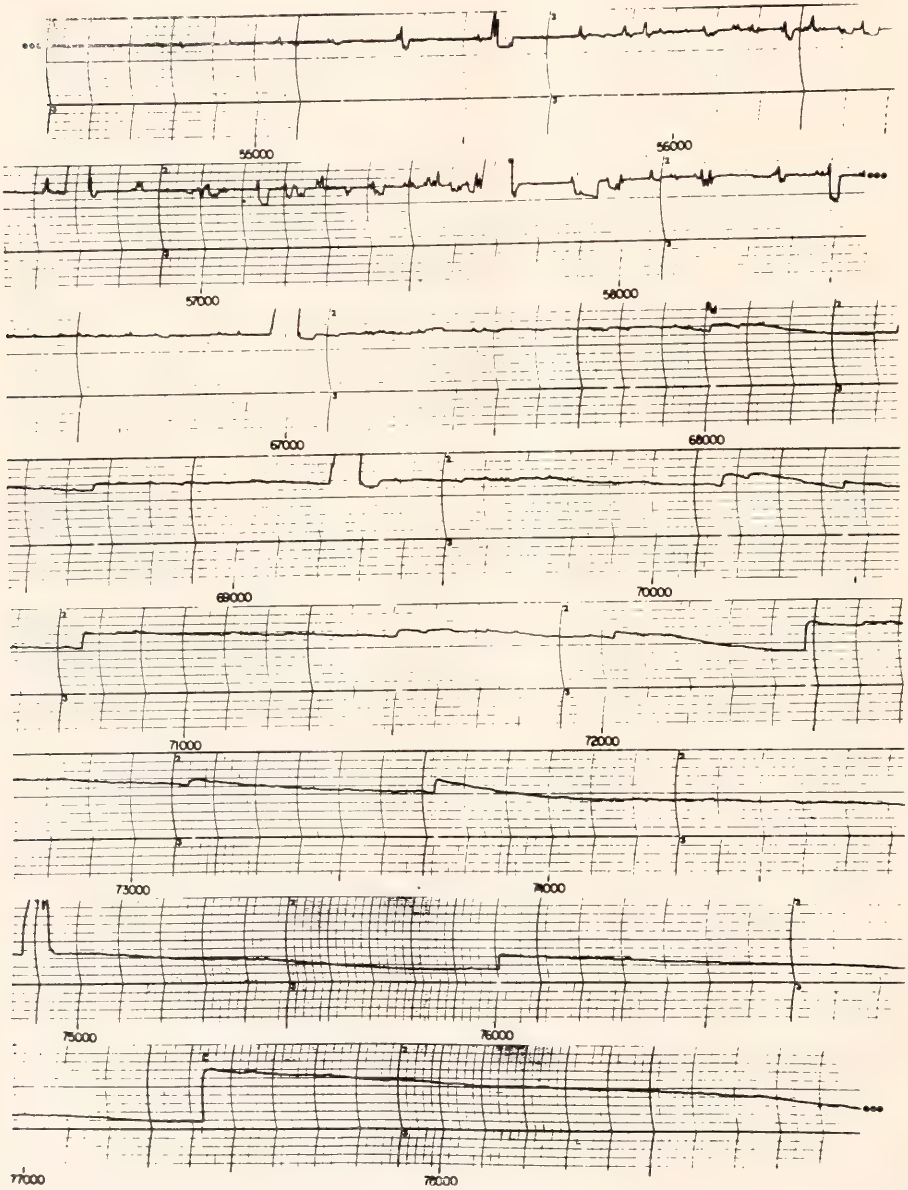

FIGURE 3. Intratracheal pressure record of pupa II during part of a burst at 0° C. A portion of the open phase (B_o) (54,500 to 68,000 seconds) and the entire decline phase (B_d) (68,000 to 77,400 seconds) are shown. Note the microcycles in the decline phase, for example, the one beginning at 76,000 seconds and ending at 77,420 seconds.

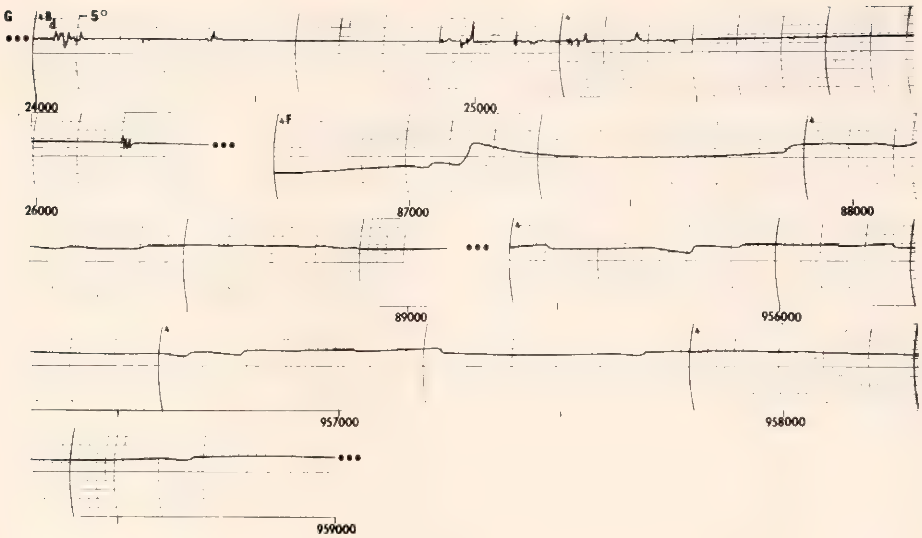

FIGURE 4. Intratracheal pressure record of pupa VI at −5° C. Since the cycle lasted at least 11 days, only part of the decline phase and a portion of the flutter period are shown. (Chart speed = 1 mm/sec.)

at 15° C it was 18%, whereas at 5° C it was 60% of the burst. At 15° C and below, the decline phase was marked by microcycles like those occurring during flutter.

The proportion of the cycle occupied by the constriction period did not change appreciably between 25° and 10° C, but at 5° C constriction was proportionally longer (45% of the cycle) than at other temperatures. The intratracheal vacuum that developed during constriction increased steadily from −1.99 mm Hg at 20° C to −5.24 mm Hg at 5° C, and then decreased to −3.13 mm Hg at 0° C (Table I).

Above 0° C the flutter period occupied a greater proportion of the cycle than either the constriction period or the burst, except at 5° C where the flutter and the constriction periods were about equally long. In two cycles at 0° C the decline phase of the burst was followed not by constriction but by the flutter period; however, flutter was interrupted by periodic bursts. In another pupa the cycle at 0° C consisted of the usual burst, constriction, and flutter periods. Although no complete cycle was recorded at −5° C, recordings of intratracheal pressure and valve behavior were begun during the early decline phase; like most cycles at 0° C, no constriction period was observed. The decline phase was followed by flutter which persisted for at least 11 days when the experiment was ended.

Changes in microcycles during the flutter period

Although valves open and close through a flutter period, they are closed most of the time (Schneiderman, 1960). When the valves open, intratracheal pressure rises to near atmospheric; when they close, intratracheal pressure falls. These fluctuations in intratracheal pressure are microcycles. At 20° C an average microcycle lasted about 53 seconds but the duration varied from 10 to 160 seconds (Table II).

Table II shows that as the temperature decreased, the average duration of microcycles in the flutter period increased. There was wide variation in the duration of microcycles at a given temperature, particularly low temperatures. However, Table II shows that the longest microcycles occurred at the lowest experimental temperatures. For example, at − 5° C a microcycle lasting 7330 seconds (2 hours) was observed whereas at 20° C the longest microcycle was 160 seconds. On the other hand, relatively brief (60 seconds) microcycles occurred at − 5° C as well as at higher temperatures.

Microcycles occurring just before the onset of a burst appeared to be shorter and to result in a smaller intratracheal vacuum than those at the beginning or middle of the flutter period. To determine whether these differences were statistically significant, the duration and the size of the intratracheal vacuums in different parts of the flutter period were measured. The first ten microcycles at the beginning, the ten in the middle, and the last ten just before the onset of a burst were chosen in each of three cycles of one pupa at 5° intervals between 20° and 0° C. The results are shown in Tables II and III.

At a given temperature the length of microcycles was not significantly different (at the 0.05 level) in different parts of the flutter period. When the same parts of the flutter period were compared at different temperatures, there were no significant differences in the length of microcycles at 20° as compared with 15° C, or at 15° as compared with 10° C. However, below 10° C the duration of microcycles in a given part of the flutter period increased significantly between 10° and 5° C, and between 5° and 0° C.

The following general observations were made regarding intratracheal vacuums during flutter: (1) At a given temperature the vacuums occurring just before bursts were smaller than those at the beginning or in the middle of the flutter period. However, vacuums occurring before a burst at 0° C were not significantly larger than those at 20° C. (2) Vacuums occurring in microcycles at the beginning and in the middle of the flutter period were significantly larger at 0° than at 20° C. (3) There was a wide range in the sizes of intratracheal vacuums occurring during the flutter period at a given temperature; however, the largest vacuums during flutter occurred at low temperatures (Table III).

Effects of low temperatures on the behavior of spiracular valves

To determine how low temperatures affect the behavior of the spiracular valves, a pupa was exposed to different temperatures, and records of intratracheal pressure and movements of the third, fourth, and fifth right abdominal spiracular valves were made.

As the ambient temperature decreased, the average number of valve movements during the flutter period decreased from 32 per minute at 20° C to 1 per minute at 0° C (Table I). At − 5° C the number decreased even further, and sometimes valve movements occurred only once an hour. However, even at low temperatures there were periods when valves moved more frequently than on the average.

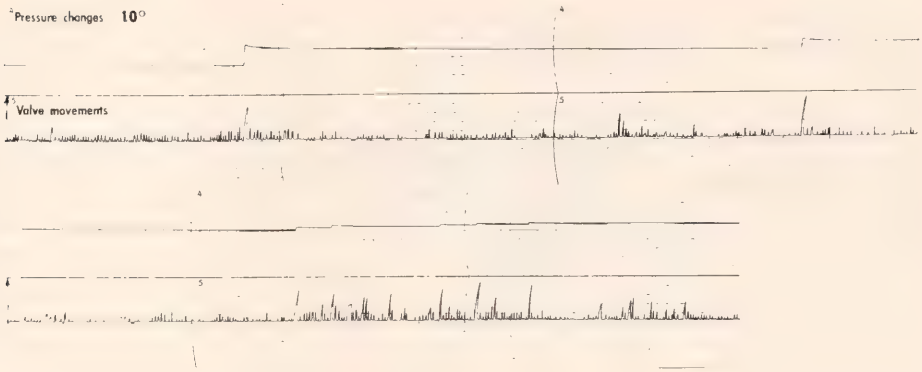

Figure 5. Simultaneous records of intratracheal pressure and behavior of 4RAS during the beginning of a pressure rise period at 10° C. Full opening of the valve is represented by a pen deflection of 5 divisions above baseline. (Chart speed = 1 mm/sec.)

Valves were capable of opening fully even at − 5° C but at this temperature opening often occurred so slowly that the movement could not be detected as it occurred. In this case movements were recorded only after it was obvious that the valve had moved.

Correlation of valve behavior with intratracheal pressure changes

Most pressure rises were accompanied by at least halfway opening of the fourth right abdominal spiracle, the spiracle we usually observed in all these experiments, although not every wide valve opening led to a pressure rise. Records of flutter occasionally showed that the valve of the fourth spiracle frequently began opening before a pressure rise occurred. However, many pressure rises were accompanied by, rather than preceded by, valve opening of the fourth right spiracle.

During the open phase of a burst at all temperatures, the valve of the fourth spiracle remained open except for occasional partial closures, so brief that the intratracheal pressure scarcely wavered from atmospheric. During the late portion of the decline phase of the burst, the intratracheal pressure records were very similar to those made during the flutter period; there were discrete microcycles, and the beginning of most microcycles was accompanied by wide opening of the valve of the fourth spiracle. However, not every wide opening was accompanied by a pressure rise.

During the constriction period, the valve of the fourth spiracle remained closed and motionless; at the same time the intratracheal pressure fell steadily. Constriction was followed by a step-wise pressure rise period (Fig. 5). As this figure shows, seven of the eight increments in pressure rise were accompanied by opening of the valve at least halfway.

Responses to oxygen and carbon dioxide

To determine what the spiracular valves do at temperatures below − 5° C, the following experiments were performed:

Ten pupae (5 per group) were weighed individually and then were placed immediately into one of two 650 ml desiccators, each containing a desiccant, $CaCl_2$ (Drierite) and a carbon dioxide absorbent, $Ca(OH)_2$ (Ascarite). Group I was kept at − 2° C and Group II at − 16° C for one week. At − 16° C the pupae were frozen and their blood was solid. Pupae then were removed individually from the desiccators and were reweighed. The average weight loss of pupae in Group I was 1.29 ± 0.56 (S.D.) % of the original weights; the average weight loss of pupae in Group II was 0.65 ± 0.29%, or about half that of pupae in Group I. Since the spiracles are the major site of water loss, the valves of pupae at − 2° C must have been open more often than those of pupae at − 16° C.

The desiccator containing the Group II pupae then was flushed at room temperature with a mixture of 20% CO_2 + 80% air to open all spiracular valves of the pupae. The desiccator, now containing 20% CO_2, again was placed at − 16° C for one week. The average weight loss following this treatment was 1.44 ± 0.65%, or 2.2× the average weight loss of the same pupae at − 16° C in air. When the spiracular valves were opened and the pupae subsequently frozen, pupal weight loss at low temperatures, *i.e.,* − 16° C, was comparable to pupal weight loss at higher temperatures, *i.e.,* − 2° C.

The average weight loss of two other groups of pupae (III and IV) in air at − 16° C was determined. Then, without permitting warming of either desiccator, the one containing Group III was flushed for 5 minutes with air while the desiccator containing Group IV was flushed simultaneously with 20% CO_2 + 80% air. Both desiccators were returned to − 16° C. When these pupae were reweighed at the end of one week, those in Group III had lost an average of 0.40 ± 0.20%, and pupae in Group IV an average of 0.22 ± 0.13% of their weight of the previous week. Pupal weight loss, even in an atmosphere in which the carbon dioxide concentration was high, was comparable to weight loss in air at the same temperature (0.40% and 0.37% for Groups II and IV, respectively). Thus it appears that the spiracular muscle does not respond to high carbon dioxide concentrations at low temperatures (− 16° C). Possible explanations for failure of the valves to respond to carbon dioxide at low temperatures are considered in the Discussion.

Direct observations of the spiracular valves and recordings of movements of the fourth spiracle revealed the following: Valve movements occurred at all experimental temperatures between ± 20° and − 5° C. No movements were observed at − 10° C or below. When 20% CO_2 + 80% air was perfused through the tracheal system at temperatures between + 20° and 0°, the fourth spiracle and its adjacent controls opened fully within 5 minutes after perfusion was begun, and remained open and motionless for the duration of perfusion. At − 5° C these valves were about three-fourths open after 5 minutes of perfusion, but full valve opening could not be elicited at − 10° C or below even when 50% CO_2 + 50% air was perfused through the tracheal system for one hour. This result indicates that at − 5° C or below the spiracular muscle does not respond to carbon dioxide.

To determine whether the spiracular response at − 5° C is solely to carbon dioxide or if the spiracular mechanism also responds to oxygen, 20% CO_2 + 80% air was perfused through the tracheal system. Ten minutes after perfusion was begun, the valves were three-fourths open and motionless. At this point, with the valves still open, the temperature was lowered to − 10° C, and an air perfusion replaced the 20% CO_2 + 80% air flow. The air flow was continued for 17 hours with no change in the position of the valves. Nor did a perfusion of 100% O_2 for two hours elicit valve closure. Then the pupa, receiving an intratracheal air perfusion, was allowed to warm to − 5° C; about seven hours later the valves closed.

In a second approach to the same question, intratracheal perfusions of both 1 and 0.5% O_2 (balance N_2) elicited opening of the valves about 20 minutes after the flow was begun at − 5° C.

The spiracular mechanism is capable of responding to intratracheal oxygen and carbon dioxide at − 5° but not at − 10° C or lower.

Discussion

Results of the present studies show that cycles of discontinuous respiration in Cecropia pupae persist at −5° C but not at − 10° C. These results confirm and extend those of Brockway (1964) and Levy and Schneiderman (1966) but contradict those of Kanwisher (1966). Since discontinuous respiration depends on the responses of the spiracular nerve and muscle to oxygen and carbon dioxide, neuromuscular activity must also persist down to − 5° C.

As expected, the length of the respiratory cycle at low temperatures was greater than at high temperatures. At − 5° C, for example, the length of the cycle was at least 50 times greater than at 20° C even though two factors, a decrease in the CO_2-trigger threshold and an increase in the length of microcycles during the flutter period, tend to shorten the cycle. At low temperatures the CO_2-trigger threshold (the concentration of carbon dioxide that causes prolonged opening of all spiracular valves) decreases (Levy and Schneiderman, 1966), and this means that less carbon dioxide than usual is required to initiate a burst. On the other hand, an increase in the length of microcycles during the flutter period means that the proportion of the flutter period during which some out-diffusion of carbon dioxide can occur, *i.e.*, the pressure rise period of microcycles, is much less at 0° than 20° C. Brockway and Schneiderman (1967) estimated that if the average length of a microcycle were 25 seconds, the spiracular valves are open no more than 8% or, more likely, 5% of the flutter period. Since an average microcycle at 0° C may be 10 times as long as at 20° C, more carbon dioxide is retained at lower temperatures than at 20° C (*cf.* Schneiderman and Williams, 1955).

Since the length of the respiratory cycle increased at low temperatures, the combined effects of a reduced CO_2-trigger threshold and the increased length of microcycles clearly were offset by other factors. The key factor is that at low temperatures the metabolic rate of insects is lower than at high temperatures. Consequently, carbon dioxide production is depressed, and a longer time elapses before carbon dioxide reaches the CO_2-trigger threshold, reduced though it may be. In addition, more carbon dioxide dissolves in tissue fluids at low than at high temperatures, and this also increases the time required for carbon dioxide to reach the triggering threshold.

Significant changes occurred during the various phases of the cycle at low temperatures. One of the most noticeable changes occurred during the decline phase of the burst. Not only did the decline phase increase in length from an average of 9 minutes at 20° C to 51 minutes at 5° C (Table I) but also it was detected from intratracheal pressure records at 15° C or below, but not at 25° or 20° C. A burst ends when the tracheal carbon dioxide falls to a minimum level (Schneiderman, 1960). Although tracheal oxygen quickly approaches ambient soon after a burst begins, the valves remain wide open for some time before they begin fluttering for an additional period (the decline phase), presumably because the pupa requires time to "unload" its accumulated carbon dioxide.

One possible explanation for prolonged valve openings during bursts at low temperatures is that the rate of carbon dioxide release is limited by the activity of carbonic anhydrase. Less than 15% of the carbon dioxide released during a burst by diapausing *Agapema* pupae is supplied by carbon dioxide in the tracheae at the onset of a burst (Buck and Keister, 1955, 1958), and this release occurs instantaneously. The remaining carbon dioxide escapes relatively slowly from the tissues during the remainder of the burst. There is enough carbonic anhydrase in the tissues of Cecropia pupae to account for the volume of carbon dioxide released during a burst (Buck and Friedman, 1958), and at low temperatures there is a decrease in the rate at which the enzyme acts. Thus, a pupa requires many minutes to unload carbon dioxide since the animal releases 2 to 3 times as much carbon dioxide during a burst at low temperatures as at higher temperatures (Schneiderman and Williams, 1955). Both the slow rate at which carbon dioxide is released and the larger volume of carbon dioxide that is impounded at low temperatures cause the valves to remain open for long periods.

There are three noteworthy points about the decline phase at low temperatures: (1) The initial part of the decline phase cannot be detected from intratracheal pressure records. (2) Fluttering and microcycles occur in later parts of the decline phase. (3) Most important, these flutters and microcycles, which constitute a second period of discontinuous respiration, persist even though the concentration of oxygen in the tracheae is close to ambient (20%). At first this is surprising since it is a *low* concentration of oxygen, *i.e.*, 5%, that normally triggers fluttering (Schneiderman, 1960; Burkett and Schneiderman, 1974).

Why do microcycles appear during the decline phase at a time when the tracheal concentration of oxygen is much higher than that which normally triggers fluttering? If carbon dioxide is indeed released slowly at low temperatures, the spiracles are still under the influence of carbon dioxide during the decline phase. The O_2-flutter threshold (the concentration of oxygen at which fluttering begins) can be raised by increasing the carbon dioxide concentration (Levy and Schneiderman, 1966; Burkett and Schneiderman, 1967, 1974). Thus fluttering can be induced even though the oxygen concentration in the tracheae is about 20% if the carbon dioxide concentration is about 7% (Burkett and Schneiderman, 1974), as it is during the decline phase (Levy and Schneiderman, 1966). Hence one expects fluttering during the decline phase.

Although the constriction period was proportionally longer at 5° C than at other temperatures (Table I), it disappeared in some cycles at 0° C and never was observed at − 5° C. Both the existence and the duration of the constriction period depend on the metabolic rate of the pupa. The size of the intratracheal vacuum, which was also greater during constriction at 5° C than at other temperatures, depends not only on the metabolic rate but also on changes in the volume of the tracheal system with intratracheal pressure changes, and on the rate at which air leaks into the tracheal system (Schneiderman, 1960; Brockway and Schneiderman, 1967). At 5° C the metabolic rate of pupae still was high enough to cause them to use oxygen more rapidly than air leaked into the tracheae; as a result, a large intratracheal vacuum developed.

In two cycles at 0° C the decline phase led directly into a flutter period instead of the usual constriction period. The reason was this: Since the metabolic rate of the pupa was quite low, the rate at which the animal used oxygen was only slightly greater than that at which air leaked into the tracheae. Thus a significant vacuum, *i.e.*, one that would facilitate the mass transfer of air past the valves, could never develop. Eventually the intratracheal oxygen concentration dropped to the level that triggers fluttering and microcycles. The presence or absence of the constriction period at low temperatures depends on metabolic rate; pupae with a sufficiently high metabolic rate will have a constriction period, and those that have a lower metabolic rate will not.

The flutter period generally lengthened as the temperature decreased. This was as expected since the metabolic rate also decreased. However, at 5° C the flutter period and the constriction period were about the same length. A possible explanation for this is that the large intratracheal vacuums which developed during both the flutter and the constriction period at 5° C impeded out-diffusion of carbon dioxide; consequently, more carbon dioxide than usual accumulated. Thus the time required to reach the CO_2-trigger threshold decreased, and the flutter period was shortened.

The flutter period is a series of microcycles which generally became longer as the ambient temperature decreased (Table II). This, too, was expected since the duration of microcycles, like that of the constriction period, depends on the metabolic rate. When a pupa's metabolic rate is low, the rate at which it uses oxygen decreases. Thus a longer time is required for the concentration of oxygen in the tracheae to drop to the O_2-flutter threshold than when the metabolic rate is high.

In the present experiments the size of the intratracheal vacuum developed during flutter also increased except at − 5° C where it was less than at 0° C. During flutter at 25° and 20° C valves are constricted most of the time. At lower temperatures down to 0° C they are constricted even more since there are fewer openings per unit time than at higher temperatures. Consequently, larger vacuums can develop during the flutter period at lower than at higher temperatures. At − 5° C the average size of the intratracheal vacuum developed during flutter was less than at 0° C, presumably since the rate of oxygen uptake at − 5° C was so low that air leaked into the tracheae almost as rapidly as oxygen was removed.

During microcycles near the end of the flutter period the size of the intratracheal vacuum was less than in other parts of the flutter period (Table III). Observations of the spiracular valves explain why (Schneiderman, 1960). Before a burst, valves begin opening and closing more often and opening more widely than in other parts of the flutter period. As a result, intratracheal pressure never has an opportunity to fall much below atmospheric.

Most movements of the spiracular valves are not effective (Brockway and Schneiderman, 1967), that is, valves may move yet they may not open the tracheal system to the atmosphere or else they open it so briefly that no pressure change occurs. The results of the present experiments confirm this. Changes in intratracheal pressure were accompanied by openings and closings of valves. However, most valve movements, especially at low temperatures, were pulsations—movements not strong enough to break the valve seal or to permit more than very small, very brief openings—and did not cause changes in intratracheal pressure.

Two factors must be remembered when attempting to correlate valve movements with intratracheal pressure changes: (1) If one spiracle closes, this does not necessarily mean that intratracheal pressure will fall since other valves may be open. (2) The fourth spiracle, which we observed, was covered with a plastic window; although the spiracle may have opened, it did not open the tracheal system to the atmosphere. The contralateral spiracle, which was not covered with a window, usually behaves in a similar manner but the precise movements of the two contralateral valves may not be exactly the same (Brockway and Schneiderman, 1967); in fact, about 20% of the time one of the two opens while the other may move slightly but does not open. Hence a brief opening in the fourth right spiracle may not have led to a synchronous pressure rise since the fourth left spiracle may not have opened at the same time. However, if the valve of the fourth right spiracle remained open for several seconds, other valves usually were also open.

There are several possible reasons why valves apparently do not respond to oxygen and carbon dioxide at temperatures below − 5° C: (1) The spiracular muscle itself may be insensitive to high carbon dioxide and to low oxygen concentrations. (2) The central nervous system, the principal target of oxygen (Burkett and Schneiderman, 1967, 1974) may not function; hence nervous control of spiracular activity may be blocked. (3) The enzymes that release the energy needed by a contracting muscle may not function at a level consistent with the energy requirements of the spiracular muscle (*cf.* Richards, 1958). (4) The muscle may be too viscous to move (*cf.* Richards, 1958). (5) The fluid film that covers the valves may become viscous, causing them to "stick." (6) The muscle may be frozen.

Which, if any, of these alternatives is responsible for the failure of the muscle to respond to oxygen and carbon dioxide is not known. However, the blood of pupae that were chilled to − 10° C under the conditions of our experiments (cooling rate $\leqslant 0.5°$ C/min) was frozen (Burkett and Schneiderman, 1968). This may also occur in nature.

The fact that discontinuous respiration and response of spiracles to oxygen and carbon dioxide were observed at − 5° C indicates that neuromuscular control of spiracular behavior persisted at this temperature. Although the spiracular muscle is innervated by a nerve from the central nervous system (Beckel, 1958), and is controlled in part by the nerve (Burkett and Schneiderman, 1974), Van der Kloot (1963) found that spontaneous potentials generated in the isolated spiracular muscle cause it to contract. He suggested that when the temperature is very low, it is these spontaneous potentials that keep the muscle contracted. Our results suggest that both the spiracular nerve and muscle are "turned off" at the same temperature or certainly within a few degrees of each other.

Fluttering is a significant mechanism by which diapausing pupae conserve water at low temperatures. Although the absolute duration of bursts at low temperatures increases, there are fewer bursts than at high temperatures, and these two factors must offset each other in providing opportunities for pupae to lose water. In the present experiments not only did the flutter period lengthen as the temperature decreased, thus occupying most of the respiratory cycle, but also the number of valve movements during the flutter period decreased. These results support an earlier suggestion that ". . . the heart of the problem (water conservation) surely lies in the flutter period, where hour after hour the insect practices the 'trick' of filtering in oxygen, while retaining carbon dioxide and water" (Schneiderman, 1960, page 525).

At some temperature (between − 5° and − 10° C in the present experiments) discontinuous respiration ceases. At that point pupae must rely on other mechanisms of gas exchange and water conservation. Kanwisher (1966) concluded that at low temperatures, where discontinuous respiration no longer occurs, gas exchange occurs by pore diffusion. Since the pupa's respiratory demands are low ($\leqslant 0.1$ mm^3/g/hr at − 12° C (Kanwisher, as quoted in Asahina, 1966)), pore diffusion probably provides adequate gas exchange. Although less than 3% of the gas exchange at 25° C may occur through the cuticle (Schneiderman and Williams, 1955), at very low temperatures the insect's low metabolic demands may be met partially by this mechanism. But whatever the mechanism, one fact is clear: At low temperatures valves are constricted most of the time, and conditions in nature probably insure that they freeze in the constricted position. Hence the valves continue to serve the insect, enabling it to conserve water, even though they cannot respond to oxygen and carbon dioxide.

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SUMMARY

1. Experiments were performed to examine the effects of temperatures down to − 20° C on discontinuous respiration in diapausing Cecropia pupae. Methods were developed that permitted simultaneous recording of intratracheal pressure changes and spiracular valve movements at low temperatures.

2. Discontinuous respiration continued down to − 5° C but ceased at some temperature between − 5 and − 10° C.

3. At 0° C the constriction period of the cycle, during which valves remain closed for some time, generally was absent. The open phase of the burst was followed by a lengthy decline phase, during which valves close briefly from the fully open position, and then by the flutter period, during which valves continuously open briefly and then close. The flutter period made up most of the cycle at all temperatures except at 5° C where the flutter and constriction periods were of about equal length.

4. Longer microcycles, relatively brief pressure fall periods caused by the closing of the spiracular valves, with correspondingly greater intratracheal vacuums than at high temperatures were observed in the decline phase of the burst and in the flutter period as the ambient temperature decreased to 0° C. At − 5° C

microcycles were shorter and resulted in less of an intratracheal vacuum than at 0° C, presumably because the metabolic rate of the pupa decreased.

5. The spiracular valves responded to oxygen and carbon dioxide at − 5° C but not at − 10° C or below. Thus neuromuscular coordination of spiracular function persisted at − 5° C but ceased at some temperature between − 5° and − 10° C.

6. Studies of pupal weight loss indicated that spiracular valves remained constricted at low temperatures. Under the experimental conditions pupae froze at some temperature between − 5° and − 10° C. It is suggested that in nature the spiracular valves freeze in the closed position.

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IDENTIFICATION AND CHARACTERIZATION OF LYSOZYME FROM THE HEMOLYMPH OF THE SOFT-SHELLED CLAM, *MYA ARENARIA*[1]

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Reactions in pelecypod molluscs to experimentally or naturally introduced nonself materials, biotic or abiotic, is primarily cellular, *i.e.*, if the foreign material is too large to be phagocytosed, it is encapsulated (see Feng, 1967; Cheng, 1967; and Cheng and Rifkin, 1970 for reviews). In the case of materials small enough to be phagocytosed, those which are digestible usually are degraded intracellularly (Tripp, 1958a, 1958b, 1960; Feng, 1959, 1965). That the vegetative cells of certain bacteria are reacted against in this manner has been documented by electron microscopy (Cheng, Cali and Foley, 1974; Cheng and Cali, 1974). Consequently, it appeared to be of interest to determine whether lysozyme and other enzymes occur in the hemolymph of several species of pelecypods and if so, to ascertain their kinetic properties. Such studies directed at the identification and characterization of lysozyme have been carried out on the serum and leucocytes of the American oyster, *Crassostrea virginica*, by Rodrick and Cheng (1974) and it has been shown that this bacteriolytic enzyme does indeed occur in both the serum and leucocytes of that mollusc. Thus, the earlier reports by McDade and Tripp (1967a, 1967b) have been confirmed and in addition, we have characterized the oyster lysozyme relative to its stability to heat, sensitivity to changes in ionic concentration, salt dependency, optimal pH range, and activity on selected species of bacteria.

The second species of pelecypod that has been studied is the soft-shelled clam, *Mya arenaria*, and in this paper is reported our studies on the lysozyme of this mollusc.

MATERIALS AND METHODS

Collection of whole hemolymph

The soft-shelled clams, *M. arenaria*, used in this study were collected from the vicinity of Sandy Hook, New Jersey, and were maintained for up to 40 days in recirculating seawater tanks with a salinity of 25‰. During this period, hemolymph samples were taken from the mantle cavity, heart, and pericardial sac by use of sterile Pasteur pipettes. Because all pelecypods possess an open circulatory system, all hemolymph samples taken from these sites are assumed to be identical relative to qualitative composition.

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Measurement of lysozyme activity

Lysozyme (EC 3·2·1·17, N-acetylmuramide glycanohydrolase) activity in the hemolymph of *M. arenaria* was determined spectrophotometrically by a modification of the method of Shugar (1952). The modification involved using 0.2 mg/ml of dried *Micrococcus lysodeikticus* cells (Sigma, St. Louis, Missouri) and 0.1 M glycine buffer at pH 5.5. All reactions were initiated by the addition of hemolymph, *i.e.*, enzyme, and measured by use of a Gilford 240 spectrophotometer equipped with a Model 6040-A heat writing recorder. Only the initial velocities were measured and in all cases the initial velocities doubled when the amount of enzyme (hemolymph) was doubled.

The protein concentration of whole hemolymph was determined by the method of Lowry, Rosebrough, Farr and Randall (1951) and crystalline bovine serum albumin (Sigma) was used as the standard.

Determination of pH optima

In order to determine the pH optima in the presence of several buffers, assays for lysozyme activity were carried out at various pH's in the presence of saturating substrate levels of 0.2 mg ml using the following four buffers: (1) 0.1 M glycylglycine at pH 4.0 to 9.0, (2) 0.1 M imidazole at pH 4.0 to 9.0, (3) 0.1 M Tris-HCl at pH 4.0 to 9.0, and (4) 0.1 M phosphate at pH 4.0 to 9.0. The pH of the lysozyme reaction mixture was determined before, during, and after each enzyme reaction.

Determination of end products

The lysozyme reaction mixtures were analyzed qualitatively for the presence of amino sugars (Rondle and Morgan, 1955) and reducing sugars by use of Benedict's solution (McDade and Tripp, 1967b) before, during, and after enzyme reactions.

Thermal stability of lysozyme

To determine the effect of temperature on the enzyme, samples of fresh, whole hemolymph were incubated at temperatures from 10° C to 100° C at 10° intervals for 30 minutes before being assayed for lysozyme activity.

Distribution of lysozyme

Fresh, whole hemolymph was centrifuged at 4000 × *g* for 15 minutes and the resulting pellet was homogenized in a minimal amount of 0.25 M, 0.5 M, or 0.9 M sucrose, whole hemolymph, or sea water that had been passed through a 0.22 μm millipore filter. The crude homogenate was subsequently recentrifuged for 10 minutes at 4000 × *g*. The supernatant was decanted off and recentrifuged at 10,000 × *g* for 30 minutes. This was done to pellet any organelles that had been released during homogenization. All of the supernatants and pellets were tested for lysozyme activity and the protein content of each constituent was determined.

Effect of lysozyme on bacteria

To test the specificity of the lysozyme in *M. arenaria* hemolymph, seven species of bacteria in addition to *Micrococcus lysodeitikus* were tested against this enzyme. These bacteria were *Bacillus megaterium, B. subtilis, Proteus vulgaris, Salmonella pullorum, Shigella sonnei, Escherichia coli,* and *Staphylococcus aureus.* The procedure followed is identical to that described for *M. lysodeitikus*, which involved determining alterations in optical density of the enzyme-bacteria mixtures.

Reactivation of lysozyme by salts

Freshly collected hemolymph from *M. arenaria* was dialyzed in 0.1 M glycylglycine buffer at pH 5.5 for 210 minutes after which the lysozyme activity was determined in the absence and presence of various concentrations of NaCl, KCl, and $MgCl_2$.

The sigmoid portions (first halves) of the salt reactivation curves were analyzed by use of a Hill plot to determine the minimal number of interacting binding sites for the salts during reactivation of the lysozyme.

Using the Michaelis-Menten assumption of equilibrium kinetics, *i.e.*,

$$v_0 = \frac{V_m (s)^n}{K_m + (s)^n}$$

where v_o is the initial velocity, s is the salt concentration, and n is the number of interacting binding sites, and by taking the logarithmic form of the equation given above and arranging the logarithmic expression for the equation to fit a straight line ($y = mx + b$), the following equation is obtained:

$$\log (v_o/V_m - v_o) = n \log (s) - \log K_m$$

When the data are plotted using the coordinates $\log (v_o/V_m - v_o)$ *vs.* $\log (s)$, the resulting Hill plot provides a n (= slope) equal to the minimum number of interacting binding sites (Atkinson, Hathaway and Smith, 1965).

Effect of heavy metals on lysozyme

In order to determine whether heavy metals inhibit the lysozyme from *M. arenaria* hemolymph, enzyme assays were carried out as described above but in the presence of 5 μM of zinc acetate and 0.6 μM *of* lead nitrate. In addition, since Smith and Stocker (1949) have reported that egg-white lysozyme is inhibited by sodium tartrate, we have tested the effect of 0.1 mM of sodium tartrate on the enzyme from the clam.

RESULTS

Our results indicate that the hemolymph of *Mya arenaria* includes lysozyme activity. This conclusion is based on the finding of (1) a reduction of turbidity measured at 540 mμ when intact cell walls of several species of bacteria are placed in the hemolymph, and (2) the liberation of reducing and amine sugars by intact bacterial cell walls when placed in the hemolymph.

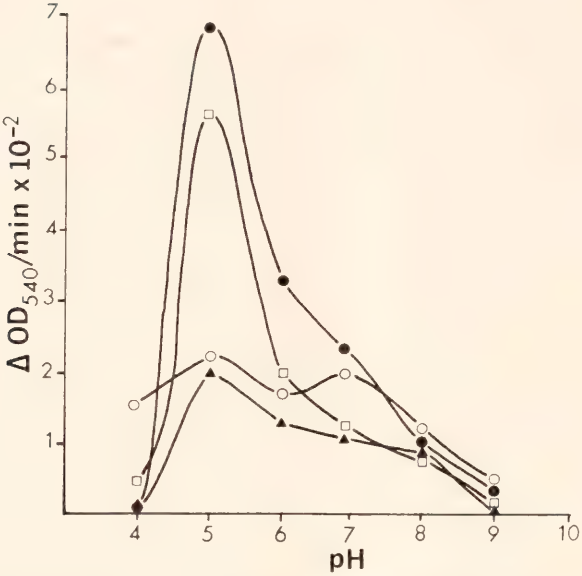

FIGURE 1. Graph showing effect of pH on the activity of the lysozyme in whole hemolymph of *Mya arenaria* using 0.1 M glycylglycine (black dots), 0.1 M Tris-HCl (circles), 0.1 M imidazole (squares), and 0.1 M phosphate (triangles) as buffers. The lysozyme activity is expressed as ΔOD_{540}/min × 10^{-2} at 25° C.

The effect of pH on the lytic activity of whole hemolymph is shown in Figure 1. When 0.1 M glycylglycine, 0.1 M imidazole, or 0.1 M phosphate buffers are used, the optimal pH of the lysozyme has been determined to be 5.0. On the other hand, the optimal pH is 4.5 when a 0.1 M Tris-HCl buffer is employed. It is noted that a sharp peak is obtained at pH 5.0 and relatively high lysozyme activities have beeen recorded between pH 4.5 and 6.0 when 0.1 M glycylglycine and 0.1 M imidazole are used. However, low activities and a relatively broad and nonspecific pH effect is observed when 0.1 M Tris-HCl and 0.1 M phosphate buffers are employed.

As indicated in Table I, the major portion of the lysozyme activity is associated with the 4000 × *g* supernatant while only a small amount of activity is associated with the 4000 × *g* pellet. Similarly, when the 4000 × *g* supernatant is recentrifuged at 10,000 × *g*, most, if not all, of the lysozyme activity is associated with the supernatant.

TABLE I

Distribution of lysozyme activity in the hemolymph of Mya arenaria

Sample	ΔOD_{540} min	Specific activity
Whole hemolymph	0.012	0.009
4,000 × g supernatant	0.020	0.014
4,000 × g pellet	0.008	0.004
10,000 × g supernatant	0.021	0.015
10,000 × g pellet	0.001	undetectable

TABLE II

Lytic activity of lysozyme in whole hemolymph of Mya arenaria *on several species of bacteria. The specific activities are reported at $\Delta OD_{540}/min/mg$ of protein at 25°C and pH 5.5*

Bacteria	ΔOD_{540} min	Specific activity
Micrococcus lysodeitikus	0.021	0.020
Bacillus megaterium	0.018	0.014
Bacillus subtilis	0.008	0.006
Proteus vulgaris	0.016	0.010
Salmonella pullorum	0.013	0.009
Shigella sonnei	0.011	0.008
Escherichia coli	0.009	0.007
Staphylococcus aureus	no activity	no activity

Eight species of bacteria were tested against the lysozyme in *M. arenaria* hemolymph. As indicated in Table II, the enzyme is most active in the degradation of *Micrococcus lysodeitikus, Bacillus megaterium,* and *B. subtilis.* There is a low level of activity against *Proteus vulgaris, Salmonella pullorum, Shigella sonnei,* and *Escherichia coli* and the lysozyme has no effect on *Staphylococcus aureus.*

That the hemolymph lysozyme of *M. arenaria* is very sensitive to dialysis is demonstrated by the reduction of its activity by 50% after 30 minutes of dialysis and the total inactivation of the enzyme after 210 minues of dialysis in 0.1 M glycylglycine (Fig. 2).

The addition of either NaCl or KCl to hemolymph that had been inactivated by dialysis restores the lysozyme activity (Fig. 3). However, the addition of both $MgCl_2$ and $FeCl_3$ has little effect on reactivation of the enzyme. It is noted that the highest lysozyme activity (ΔOD_{540}/min) is obtained with 100 mM of NaCl or KCl. Furthermore, the activation portion of the curve (first half) is sigmoidal while the deactivation portion (second half) is not sigmoidal. By plotting the data from the sigmodial activation portion as a Hill plot (Fig. 4), a straight line with a slope (n value) of 2.3 for NaCl and 2.8 for KCl is obtained. These slopes (n values) are equal to the minimum number of interacting binding sites for NaCl and KCl.

TABLE III

The effects of sodium tartrate, zinc acetate, and lead nitrate on lysozyme activity in the hemolymph of Mya arenaria

Sample	ΔOD_{540}/min	Specific activity	% inhibition
Fresh whole hemolymph	0.025	0.020	0
Fresh whole hemolymph + 0.1 mM sodium tartrate	0.008	0.006	68.0
Fresh whole hemolymph + 5 μM zinc acetate	0.011	0.008	56.0
Fresh whole hemolymph + 0.6 μM lead nitrate	0.005	0.003	80.0

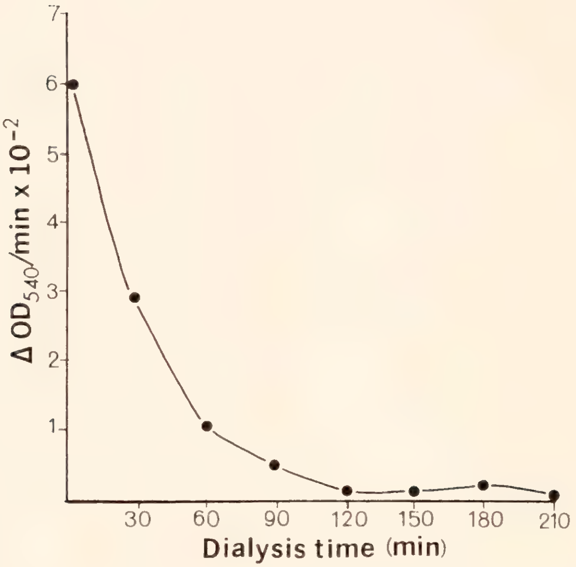

FIGURE 2. Graph showing the effect of dialysis on the lytic activity of the lysozyme in the whole hemolymph of *Mya arenaria* using 0.1 M glycylglycine at pH 5.5. The lysozyme activity is expressed as ΔOD_{540}/min × 10^{-2} at 25° C.

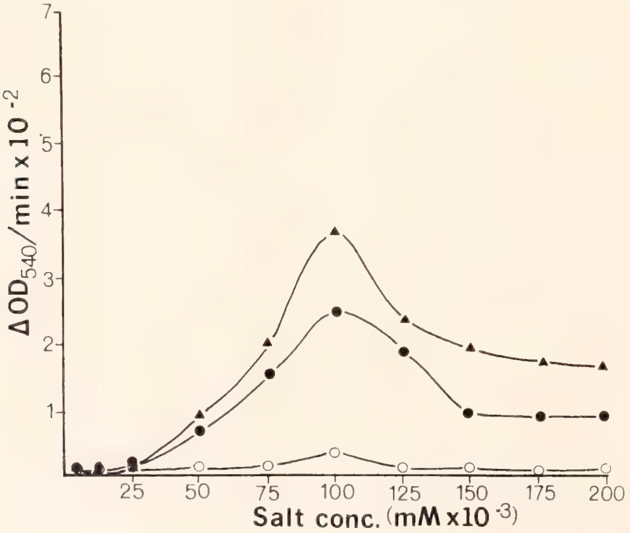

FIGURE 3. Graph showing the effect of various salt concentrations on the lytic activity of the lysozyme in the whole hemolymph of *Mya arenaria* using NaCl (triangles), KCl (black dots), and $MgCl_2$ (circles). The lysozyme activity is expressed as ΔOD_{540}/min × 10^{-2} at 25° C and pH 5.5.

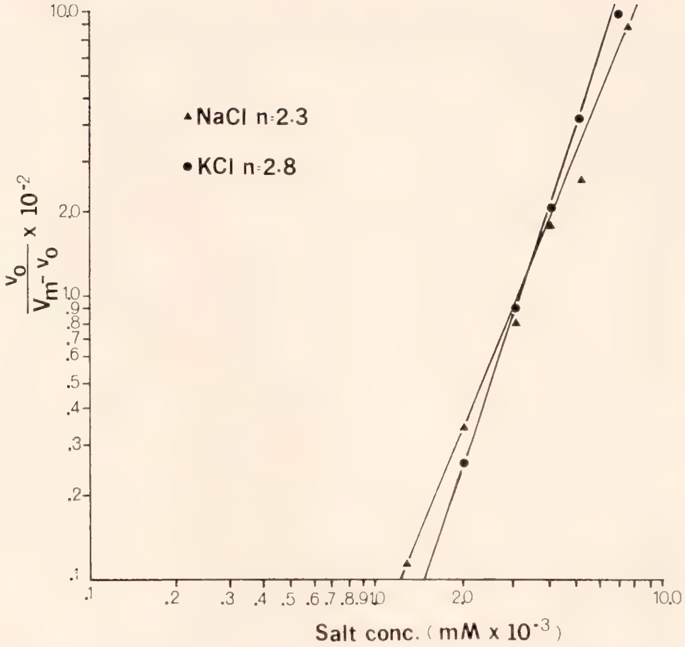

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FIGURE 4. Hill plot analysis of the effect of various concentrations of NaCl (triangles) and KCl (black dots) on the lytic activity of the lysozyme in the whole hemolymph of *Mya arenaria*. The slope (n) is equal to the minimum number of interacting binding sites.

The lysozyme in the hemolymph of *M. arenaria* is extremely sensitive to small amounts of zinc acetate, lead nitrate, and sodium tartrate (Table III). Lead nitrate is approximately 10 times more effective than zinc acetate and considerably more effective than sodium tartrate in inhibiting lysozyme activity.

DISCUSSION

According to Jollès (1964), the following criteria must be met before an enzyme can be designated as lysozyme. Specifically, (1) it must catalyze the release of reducing sugars from susceptible bacteria, (2) it must cause the liberation of amine sugars and/or muramic acid from susceptible bacteria, and (3) it must cause the reduction in turbidity of intact bacterial cell walls. The enzyme that we have examined from the hemolymph of *M. arenaria* meets these criteria and therefore can be considered as lysozyme.

The lysozyme from *M. arenaria* hemolymph shares several biochemical properties with lysozymes from other sources. It is relatively heat stable, being able to withstand 75° C for 30 minutes; is salt dependent; and portrays sensitivity to alterations in salt concentration. Relative to the last characteristic, it is noted that in the absence or very low concentrations of salts, the *M. arenaria* lysozyme has essentially no effect on the cell wall of *Micrococcus lysodeikticus* as indicated by only minute amounts of reducing and amine sugars released and little, if any, decrease in turbidity of the bacterial reaction mixtures. Based on the available data, it would appear that the salt dependency of the lysozyme is specific for monovalent cations since both KCl and NaCl are very effective in restoring enzyme activity while $MgCl_2$ is rather ineffective.

As reported, the activation of the molluscan lysozyme at low cation concentrations is sigmoidal, thus indicating the possible occurrence of preferential interacting binding sites for NaCl and KCl. A Hill plot analysis of these data has revealed that a minimum of 2.0 binding sites occur and are required for optimal activity. On the other hand, the inhibitory segments of the two salt-dependency curves (Fig. 3) are not sigmoidal and hence probably do not involve multiple reaction sites. Therefore, it may be concluded that the activation and inhibition of lysozyme activity by NaCl or KCl may represent two distinct kinetic processes. It is noted that Fitt, Dietz and Grunberg-Manago (1968), working with polynucleotide phosphorylase, have reported activation of this enzyme by low salt concentrations in a manner similar to that of the hemolymph lysozyme of *M. arenaria*. It is also noted that the hydrolysis of glycol chitin requires the presence of small amounts of salts (Rupley and Gates, 1967) and the interactions of lysozyme with chromatographic columns of neutral chitin are affected by changes in pH and ionic concentration of the medium (Davis, Heuberger and Wilson, 1969). Consequently, the overall conformation of lysozyme may require the presence of salts to affect lysis.

Phillips (1967), based on x-ray crystallography data, has shown that the lysozyme molecule includes high concentrations of salts which may suggest a role for salts in the overall confirmation of the enzyme; however, Praissman and Rupley (1968) have demonstrated that increases in ionic concentrations have only a minimal effect on the tritium-hydrogen exchange rates of lysozyme. This information could be interpreted to negate Phillips' hypothesis. Probably a more satisfactory explanation for decreased lytic activity of lysozyme in the absence of salts is that of Davis *et al.* (1969) who have postulated that the electrostatic binding between the enzyme and the bacterial cell wall occurs in either a "productive" or "unproductive" fashion similar to that of oligomers of chitin (Rupley and Gates, 1967).

It is noted that the levels of lysozyme activity are higher in both the 4000 × *g* and 10,000 × *g* supernatants than in the corresponding pellets. The latter is comprised primarily of cells and cellular constituents. Although evidence pertaining to molluscan hemolymph cells is yet unavailable, it is possible that this phenomenon reflects the release of lysozyme from cells into the serum as Wright and Malawista (1972) and Zurier, Hoffstein and Weissman (1973) have reported for mammalian leucocytes. If such is the case, then lysozyme, and possibly other lysosomal enzymes, released into the serum in molluscs may also play a role in extracellular internal defense against invading bacteria.

We wish to acknowledge Ms. Sherry Koehler for her technical assistance.

SUMMARY

Lysozyme activity has been demonstrated in the hemolymph of the soft-shelled clam, *Mya arenaria*. When whole hemolymph is centrifuged at 4000 and 10,000 ×

g and each constituent is assayed, lysozyme activity is found to be greater in the two supernatants than in the corresponding pellets.

The lysozyme from *M. arenaria* hemolymph is salt dependent, relatively heat stabile, very sensitive to alterations in ionic concentration and the presence of heavy metals, and has an optimal pH of 5.0 when 0.1 M glycylglycine, 0.1 M imidazole, or 0.1 M phosphate buffers are employed but an optimal pH of 4.5 when 0.1 M Tris-HCl buffer is used.

A Hill plot of the data resulting from salt reactivation studies indicates that the lysozyme in *M. arenaria* hemolymph includes at least 2.0 interacting binding sites for NaCl and KCl.

When tested against a number of bacteria, the lysozyme is most active against *Micrococcus lysodeitikus* and *Bacillus megaterium.* It is less active against *Proteus vulgaris, Salmonella pullorum, Shigella sonnei, Bacillus subtilis,* and *Escherichia coli.* It is not active against *Staphyloccus aureus.*

It is suggested that the lysozyme in serum may be released from hemolymph cells.

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LARVAL DEVELOPMENT OF THE GIANT SCALLOP *PLACOPECTEN MAGELLANICUS* (GMELIN)[1]

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The giant sea-scallop, *Placopecten magellanicus* (Gmelin) ranges along the east coast of North America from Labrador to Cape Hatteras (Abbott, 1954). Although primarily a continental shelf species, it may be found one meter below low tide in the Gulf of Maine (Read, 1967). At the southern end of its range, off North Carolina, *P. magellanicus* generally occurs in water over 150 feet (46 meters) deep (Porter, 1974).

A regional fishery for *P. magellanicus*, chiefly off New England and eastern Canada, has stimulated some research on the biology of this scallop including studies of length-weight relationships and gonad development (Haynes, 1966); thermal tolerances and acclimation (Dickie, 1958); and growth rates in different geographical regions (Stevenson and Dickie, 1954 and Merrill, Posgay and Nichy, 1966). Up to the present, however, research on the giant scallop has been confined to the adult stage. The dearth of information concerning larval biology of *P. magellanicus* has formed a conspicuous gap in knowledge of the life history of this species.

The purpose of this paper is to describe the complete larval development of *P. magellanicus*, including observations on larval salinity tolerance and settling behavior. Information derived from rearing larvae in the laboratory may help to confirm the identification of *P. magellanicus* larvae from the plankton and will aid future mariculture efforts with this valuable food species.

MATERIALS AND METHODS

Sexually mature scallops were collected by SCUBA diving at Isles of Shoals, New Hampshire on September 21, 1973. They were placed in sea water in a styrofoam chest and held for 8 hours at approximately 5° C until returned to the laboratory. I selected small adults (3″–5″ diameter) to minimize stresses to the animals from confinement in limited volumes of water both during transport and in subsequent spawning containers in the laboratory.

Males and females were separated for spawning, which was stimulated thermally by raising the temperature 3–5° C. Gametes and developing larvae were generally handled as recommended by Loosanoff and Davis (1963) and Culliney, Boyle and Turner, (1974).

Larvae were reared in cylindrical glass jars containing two liters of sea water filtered to remove particles larger than one micron in size. The sea water was changed every two days. Salinity during development was nearly constant at

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32‰, and was measured with hydrometers. Dilutions for the experiment on salinity tolerance were made using filtered pond water.

The naked flagellate, *Isochrysis galbana* Parke was used to feed larvae up to the settling stage. Spat have been reared using a combination of *I. galbana* and *Chroomonas salina* Taylor. Concentrations of algal food ranged from approximately 50,000 cells/ml in cultures containing the youngest veligers to 5,000 cells/ml as larvae approached the pediveliger stage. Young spat, however, were fed concentrations approximating 10,000 cells/ml.

Measurements of larvae were made using a calibrated ocular micrometer. Clean shells of pediveliger larvae were prepared by feeding live larvae to small sea anemones. After the empty shells were egested by the anemones, right and left valves were teased apart so that the hinge structure could be observed and measurements made of individual valves.

Antibiotics were not used until larvae reached the pediveliger stage. Then 50 ppm Sulmet (sodium sulfamethazine) was added to all cultures to arrest an apparent necrotic disease.

RESULTS

Spawning

One diver in our party observed spawning in the undisturbed population from which adults used in this study were taken; he reported seeing a scallop suddenly emit a puff of milky material. On the day of collection, September 21, 1973, the temperature of the bottom water (about 50 feet deep) was 14° C; that of the surface was 16° C. Many captured scallops of various sizes (2″–8″ diameter) spawned profusely in buckets of sea water within minutes after they were brought to the surface.

In the laboratory, some individuals spawned readily within minutes after the temperature was raised from 5° C to 10° C. Others were held in large bowls of aerated filtered sea water at 12° C. A number of these spawned two days later when the water temperature was raised to 15° C.

In all, two minor and two major spawnings were cultured from separate groups of individuals. The minor spawnings were not reared past the early veliger stage due to low numbers and deformities of the larvae. Each culture was produced by a different female and several males. Cultures from different spawnings were not mixed; several populations of a convenient two-liter size were set up from each of the two major spawnings.

Development of larvae

The main features of development are summarized in Table I. Development to the earliest swimming stage occurred at 12° C in all populations and lasted between 30 and 40 hours. The embryos first became motile as ciliated gastrulae averaging 69 microns long and 63 microns across the greatest diameter. They appeared to elongate slightly into a trochophore-like stage, then became typical shelled straight-hinge veligers (Fig. 1) on the fourth day after spawning. Young straight-hinge larvae possessed a very short apical flagellum. The mean size of the earliest straight-hinge larva was 105 microns long by 82 microns high. The hinge line averaged 81 microns. (In the following description, shell dimensions will be given as length × height in consistent order.)

Development from the ciliated gastrula to the straight-hinge stage occurred over the temperature range 12° C to 18° C. Such a thermal gradient, arranged vertically by setting culture jars in a shallow cold-water bath, allows the motile larvae to seek their optimum temperature during the thermally sensitive period of straight-hinge shell deposition (Culliney *et al.*, 1974).

Initially several populations of veligers were reared at two temperatures, 15° C and 19° C, but populations at 19° C did not complete development. Development was rapid with little apparent difference in growth rates at the two temperatures.

TABLE I.

Major features of larval development of Placopecten magellanicus *at 15° C, 32‰*

Stage or distinctive feature	Age	Mean size (μM)	Range (μM)	S.E. (μM)	N*	Remarks
Unfertilized egg	0	64	63–65		17	Color pink to brown Temp. 12° C.
Earliest swimming stage (gastrula)	30–40 hours	length: 69 diam.: 63	68–70 62–64	0.35 0.24	6 13	Temp. 12–18 °C.
Earliest veliger (straight-hinge)	4 days	length: 105 height: 82 hinge-line: 81	99–107 77–84 75–82	0.24 0.24 0.24	27 27 27	Temp. 15° C.
Loss of apical flagellum	8 days					
Umbo veliger	13–28 days	length: height:	175–264 155–232		61 61	Temp. 15° C.
Appearance of eyespot Bottom seeking behavior Development of foot Adhesive tendency	23 days	length: >230 height: >210				
Pediveliger (sporadic crawling, weak byssus, growth of gill rudiment)	28 days	length: 279 height: 242 depth: 127	266–292 238–254 110–142	0.94 1.64 1.40	46 46 20	Temp. 15° C.
Spat (extensive crawling, strong byssus, growth of dissoconch shell and gill)	>35 days					

* N refers to the number of individuals measured.

The tiny apical flagellum disappeared; it could not be detected by the eighth day. The hinge line remained visible and of nearly constant length until about the 13th day when larvae began to exceed 175 × 150 microns in size (Fig. 1).

Beginning on the 15th day, extensive mortalities were observed in populations at 19° C. By the 19th day, cultures at this temperature were nearly completely decimated. A fungus appeared associated with the mortality at 19° C, but was not seen attacking living larvae. Throughout this period, populations of larvae at 15° C appeared normal and showed almost no mortality.

During larval development, shell length remained 25–30 microns greater than height. A regression of length *versus* height, calculated from over 100 measurements of veligers in all stages of development, illustrates the quantitative relationship between the two dimensions: height = 0.96 length − 18.2.

The extended velum in swimming larvae had a unique "keyhole" outline. This was most pronounced in half-grown veligers. The outline of the velum tended to become nearly oval as larvae approached the pediveliger stage (Fig. 2a).

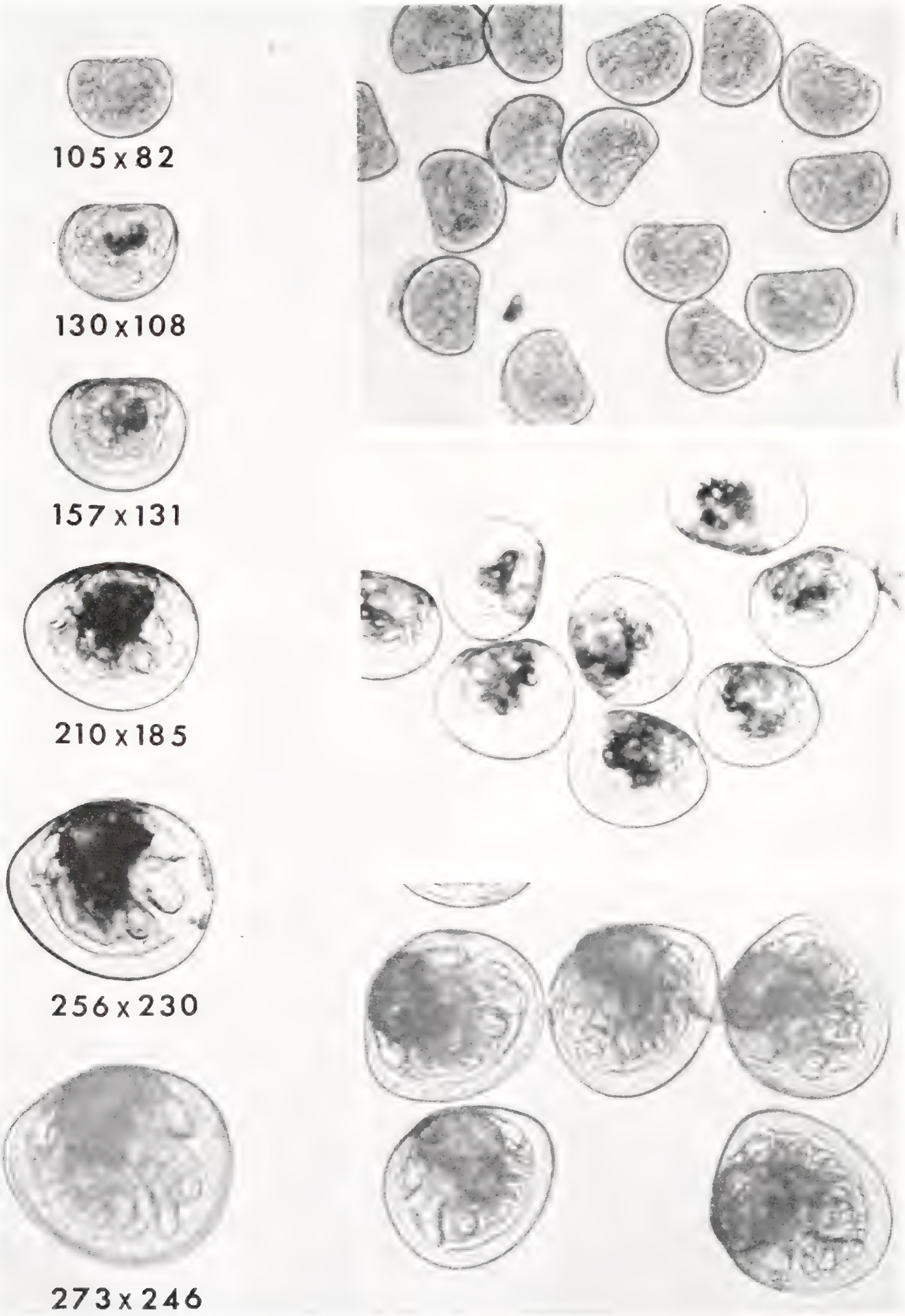

105 x 82
130 x 108
157 x 131
210 x 185
256 x 230
273 x 246

FIGURE 1. Individual and group photographs of *Placopecten magellanicus* larvae from the straight-hinge stage to the pediveliger (sizes in microns). The more pointed end, to the left in photos of individual larvae, is the anterior end.

The shape of the larval shell, standing on edge (Fig. 2b) revealed a slightly longer taper in the anterior than in the posterior direction. These features may have some taxonomic value.

On the 23rd day, typical bivalve veliger eyespots, such as occur in ostreid and mytilid larvae, were first noticed in larvae larger than 230 × 200 microns (Fig. 2c). By this time the larval foot had become prominent, although it was not yet observed extending outside the shell. In addition many larvae showed a change in swimming behavior. Prior to the 23rd day they were well dispersed in the water column, althought most swam in the upper third of the container. After the 23rd day increasing numbers of larvae were observed massed in dense swarms, swimming just above the bottom of the culture jar.

As larvae approached the pediveliger stage, they often displayed the foot as they swam. The most conspicuous feature of the foot was a cluster of long active cilia at its tip (Fig. 2d); also a well-formed heel or byssal spur was present. About this time, the larvae began to show an adhesive tendency, causing them to stick to each other and to dust particles, pseudofeces, and other debris. By the 28th day after spawning more than 50% of the larvae in all populations possessed the functional foot characteristic of the pediveliger stage. Pediveligers crawled readily, but for only short distances on glass slides and on the glass bottoms of culture containers. Crawling seemed to be initiated after larvae were disturbed. For example, brief episodes of crawling occurred when larvae were discharged from a pipette, or after they were sieved and placed in new culture containers. Pediveligers secreted a weak byssus which could not be seen readily, even when magnified at 100 ×. A prominent gill rudiment was present in most pediveligers (Fig. 2c). Average measurements of pediveliger larvae were 279 × 242 microns with a depth of 127 microns.

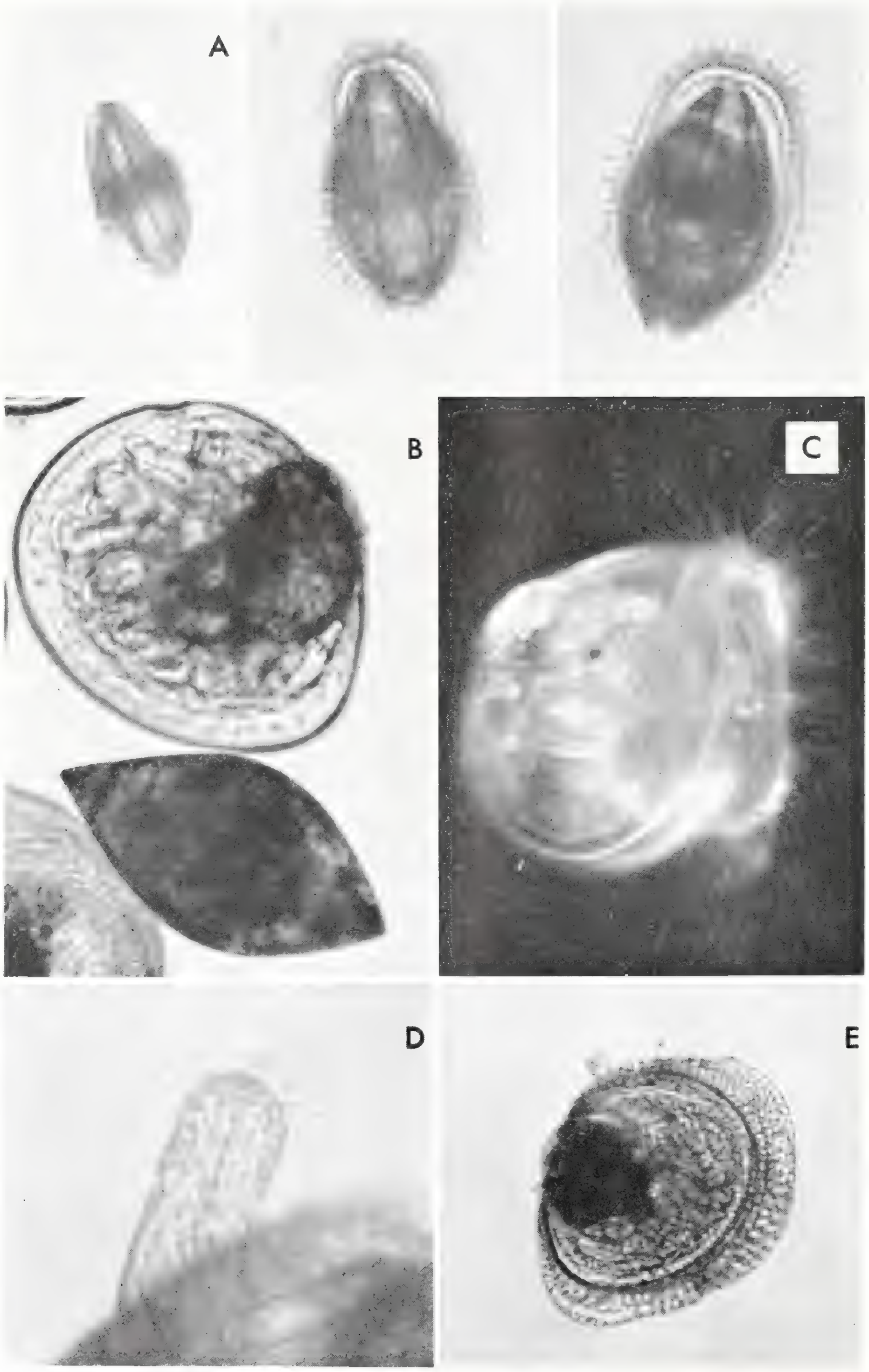
TABLE II.

Dimensions of left and right valves of Placopecten magellanicus *pediveligers*

	Left valve				Right valve			
	Mean (*μM*)	Range (*μM*)	S.E. (*μM*)	N*	Mean (*μM*)	Range (*μM*)	S.E. (*μM*)	N*
Length	280	264–292	1.64	21	276	257–290	1.76	20
Height	254	232–268	1.88	21	250	230–264	1.76	20
Depth	67	50–76	1.38	21	60	48–70	1.40	20

* N refers to the number of individual values measured.

The hinge structure of *P. magellanicus* larvae was almost featureless. A series of minute taxodont teeth were present, but their actual number was not determined and good photographs of the hinge were not obtained. Pediveliger shells were difficult to separate, indicating the presence of a strong binding force, possibly a ligament, in the hinge area. Many shells cracked or fractured in the attempt to disarticulate them. A number of shells were separated into intact valve pairs, however, and measurements of these show that the left valve averages slightly larger than the right in length, height, and depth (Table II). The difference in depth between the two valves is statistically significant at the 95% level.


A
B
C
D
E

Effects of low salinity

A mixed population of umbo-stage larvae, originating from the two female parents, was tested for salinity tolerance over a 42 hour period. Twenty larvae were pipetted into each of five finger bowls containing 180 ml of water at salinities of 10.5, 16.9, 21.5, 26.2, and 30.0‰. (Salinity was measured after the experiment). The temperature was held at 15° C. No food was given during the experiment. The LD_{50} level for salinity could not be estimated for the conditions stated, since all the larvae survived the 42 hour experiment. However, the behavior of the larvae was recorded.

At 10.5‰, larvae remained on the bottom of the finger bowl. After an initial shock produced immobility for about two hours, most larvae remained moving during the experiment. However, normal swimming was not observed. Most larvae lay on one valve and rotated in place; they appeared incapable of retracting the velum which had a distended appearance. The color of the larvae remained normal. At 22 hours, six of the twenty larvae showed no movement when the distended velum was touched by a fine needle. But at 42 hours all larvae were moving vigorously.

At 16.9‰, an initial shock produced immobility for about two hours. However, many individuals recovered and exhibited normal swimming and translational movement, but they remained close to, often touching, the bottom.

At 21.5‰, an initial shock was evident, but most larvae regained full mobility within two hours. Most larvae remained swimming close to the bottom. One individual was seen well above the bottom, however, 15 minutes after the beginning of the experiment, and two individuals swam near the surface at 42 hours.

At 26.2 and 30.0‰, no alteration of larval behavior was noted. Larvae swam throughout the water column, but most remained near the surface, throughout the experiment.

Settling behavior

The first spat was found 35 days after spawning. A conspicuous feature of the spat was the presence of a strong, thick, plainly visible byssus (Fig. 2e), contrasting with the "invisible" threads produced by pediveligers. Crawling by spat was vigorous and extensive, and was observed predominantly after the byssus was broken. At least some settling individuals cast off the velum in large pieces. On several occasions a nearly complete detached velum was seen.

P. magellanicus, however, appeared to settle in culture containers only reluctantly. As pediveliger larvae accumulated and very few spat were found, it appeared that a delay of metamorphosis was occurring. Presumably this was due to the lack of an attractive substrate for settlement. Two experiments were set up to examine potential stimuli to settlement. These included presence of adult *P. magellanicus* shell material and general thigmotatic effects of three-dimensional surfaces. The results of these experiments are shown in Figure 3.

FIGURE 2. Special features of larval development of *Placopecten magellanicus;* (A) outline of the velum of swimming larvae from early umbo stage to the pediveliger (× 120); (B) comparison of horizontal and vertical shell profiles in late umbo stage larvae (× 180); (C) gill rudiment and eyespot in pediveliger (× 140); (D) tip of pediveliger's foot, showing tuft of long cilia (× 580); (E) spat with well-formed byssus (× 110).

The experiments utilized pediveliger larvae in 4-inch finger bowls containing 200 ml of filtered sea water. Twenty larvae were exposed to each settling stimulus. Controls were simply held in finger bowls without an added stimulus. The temperature was 13°–15° C. Larvae were fed 5,000 cells/ml *Isochrysis*. Experimental and control populations were examined every 3–4 days, and fully metamorphosed and dead individuals were removed. Criteria for metamorphosis were attachment by a strong byssus and complete absence of the velum. Individuals showing only one of these features were considered partially metamorphosed (not shown in Figure 3).

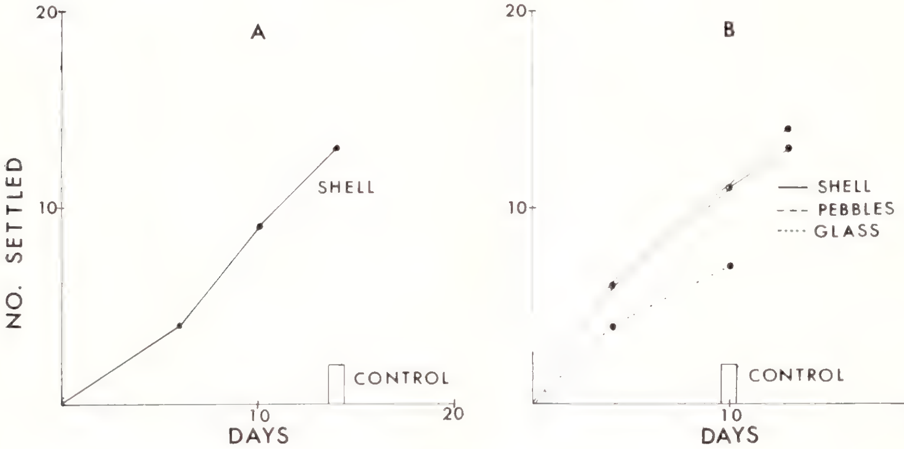


Figure 3. Cumulative plots of numbers of *Placopecten magellanicus* settling in response to different stimuli. Experiment B followed the completion of A. The single vertical bar in each plot represents metamorphosed individuals in the control populations.

Experiment A examined the reactions of larvae exposed to six fragments of fresh adult *P. magellanicus* shell. The fragments ranged from 3 mm to 2 cm long and were scattered on the bottom of the finger bowl. The results (Figure 3) show a strong settling response to the fragments. Thirteen larvae metamorphosed; two were partially metamorphosed at the end of the experiment, and three were dead. Settled larvae nearly always attached directly to the shell fragments and predominantly tended to metamorphose on the undersides of the fragments. Of the thirteen fully metamorphosed individuals, all but two were attached underneath the shell pieces, and only one did not attach to the shell. In the control, two larvae metamorphosed and one died.

An additional set of larvae was exposed at this time to increased food concentration (25,000 cells/ml *Isochrysis*. None of these larvae metamorphosed, indicating that increased food concentration is not an important settling stimulus. This result is omitted from Figure 3.

Experiment B attempted to separate properties of *Placopecten* shell as stimuli from a more general thigmotactic effect. The response to scallop shell fragments was compared to that induced by small pebbles and glass fragments. All of these objects were visibly clean but not sterile. Numbers of larvae and background conditions were the same as in Experiment A. Experiment B followed the end of Experiment A.

The results (Fig. 3) demonstrate that a strong stimulus to settling and metamorphosis is provided by all classes of physical objects tested. In the case of shell fragments and pebbles, the results were nearly identical to those in Experiment A. There is, however, a slight indication that Experiment B larvae, two weeks older than those in Experiment A, were prone to settle more quickly on the stimulus objects. Mortality in the presence of shell fragments and pebbles was low and comparable to that in Experiment A.

The population of larvae exposed to glass fragments, pieces of a microscope slide approximately the size of the shell fragments, suffered an unusually high mortality (nine dead). However, metamorphosed individuals accumulated on the glass fragmens at a rate that paralleled the results with shell and pebbles.

Again in Experiment B, most metamorphosed individuals were found on the undersides of the objects tested. In the control population only two larvae settled.

Discussion

The successful rearing of *Placopecten magellanicus* may reflect the fact that adults were obtained in prime condition for spawning. One previous attempt during the winter, to condition adults to develop gonads and spawn failed. This large active scallop may need more food than normally can be supplied under laboratory conditions. Comely (1972), working with the large European scallop, *Pecten maximus,* also experienced failure in conditioning experiments. Previous attempts by other investigators to culture *P. magellanicus* larvae have also been unsuccessful. (P. Chanley, Shelter Island Oyster Co.; H. Hidu, Univ. of Maine, personal communication).

A number of purported difficulties at the time of spawning have been encountered by investigators rearing other species of scallops. These problems have included poor vitality of embryos resulting from self-fertilization in *Aequipecten irradians* (Sastry, 1965) and *Pecten maximus* (Comely, 1972); abnormal development associated with polyspermy or supernumerary sperms (Comely, 1972 and Gruffydd and Beaumont, 1972); and mechanical damage to extremely fragile eggs by contact with a nylon sieve (Comely, 1972). Fortunately, none of these problems occurred during the rearing of *P. magellanicus.*

Abnormal larvae did appear in the two minor spawnings whose volume of eggs was 1/10 or less than that of the major spawnings. Most of the larvae from these spawnings were conspicuously smaller than larvae from major spawnings (about 10 microns in length and height at the early straight-hinge stage). However, some deformities in shell formation were also observed. It seems likely these weak spawnings consisted of unripe or underdevelopd eggs.

The mass mortality suffered by larval populations at 19° C may indicate that this temperature is close to the upper limit of thermal tolerance for larvae. Dickie (1958) found that lethal temperatures for adult *P. magellanicus* ranged from 21° C to 23.5° C, depending on acclimation. The true cause of death of the half-grown larvae at 19° C is unknown. Loosanoff and Davis (1963) observed that bivalve larvae reared at relatively high temperatures were more susceptible to mortality from disease. Gruffydd and Beaumont (1972) found that temperatures of 18° C and higher favored bacterial and ciliate growth that decimated their *Pecten maximus* larval cultures well before metamorphosis. The fungus observed attacking dead *P. magellanicus* larvae in my cultures at 19° C may have started growing in living larvae but this is uncertain.

The extremely fragile nature of larval shells of *Pecten maximus* reported by Comely (1972) was not apparent in *P. magellanicus.* Larvae were washed routinely in nylon sieves with no detectable damage to their shells from the earliest straight-hinge stage onward.

One problem, common to bivalve larvae, noted in *P. magellanicus,* was a tendency of late stage larvae to stick to each other and to debris in the cultures. This tendency appeared suddenly, at the time larvae first descended to swim in large concentrations near the bottom. The stickiness may be associated with the first attempts at byssus formation by the larvae. This interpretation is also suggested by Gruffydd and Beaumont (1972), who describe the same phenomenon in *Pecten maximus.* The problem can be controlled by drastically reducing the food allotment of the larvae when the eyespot is first detected, that is just before the appearance of bottom seeking behavior. The food-cell concentration should approximate 5×10^3 to 1×10^4 cells/cc day or less, depending on larval population density. This eliminates much of the bottom debris caused by mass wastage of excess food. Bacterial and protozoan growth is minimized. Larvae still feed efficiently with the relatively large velum at this stage and remain largely free of detritus. Newly-settled spat which climb up off the bottom should be alloted more food than pediveligers, as the tiny gill at first appears to be an inefficient feeding mechanism.

The differences in size of the right and left valves of pediveligers may have taxonomic importance. This observation, especially with respect to depth, seems to show a predisposition in larvae to the adult condition where the right valve is more nearly flat.

The observations of behavior at different salinities show that larvae might survive considerable incursion into estuaries. Also, a general lowering of salinities in coastal waters following heavy rains would not seriously affect them. Because of their bottom-seeking behavior at salinities near 20‰ and lower, larvae entering stratified estuaries might be transported some distance inland. It is also possible this behavior might indirectly cause mortalities by subjecting larvae to benthic predation or to entrapment in bottom debris before they are ready to settle.

Pediveligers appeared capable of delaying metamorphosis for at least a month. In contrast to larvae of *Mytilus edulis,* studied by Bayne (1965) *Placopecten* pediveligers did not gradually lose the velum during the period of delay of metamorphosis. Unlike *M. edulis,* the scallop larvae retained their swimming ability.

The experiments on settling behavior suggest a generalized thigmotactic response in pediveligers. This differs from the major trend of results in experiments on larval settlement in which highly specific biochemical or biophysical stimuli have been observed (Scheltema, 1961; Crisp, 1965, 1967; Wilson, 1968). Because conditions in my experiments were not sterile, there remains a possibility that larvae responded to microbial films associated with the stimulus objects. However, such stimuli should also have been present in the sea water and on the glass surfaces of finger bowls. The extremely high mortality associated with fragments of glass microscope slides may have been caused by some toxic coating on the slides, which were fresh from a new box. Attachment of spat on the undersides of the stimulus objects may be a behavioral adaptation to escape certain types of epibenthic predators, for example crabs, which are known to take a heavy toll of bivalve spat (P. Chanley, Shelter Island Oyster Co., personal communication).

A number of factors encountered in culturing larvae of *P. magellanicus* indicate that this would be a prime species for commercial mariculture. First the fact that the sexes are separate eliminates the problem of poor viability accompanying self-fertilization (Sastry, 1965; Comely, 1972). Larvae grew rapidly with little mortality at 15° C and thrived on a diet of the easily cultured *Isochrysis galbana*. Extreme precautions involving sterility of rearing equipment (Comely, 1972; Gruffydd and Beaumont, 1972) were not necessary with *P. magellanicus*, although an antibiotic such as Sulmet, used at 50 to 100 ppm would be recommended as larvae approach the pediveliger stage and aggregate near the bottom of the culture container. A reduction in food concentration would also be beneficial at this time. The natural tendency of pediveligers to settle beneath solid objects can be exploited. Spat are thus easily caught, manipulated, and transported on artificial substrates.

Presently, the most serious obstacle to hatchery production of *P. magellanicus* spat is that gametes cannot be obtained out of season. The potential for mariculture of these scallops is also a function of growth of juveniles and adults under artificial and captive conditions. New approaches and techniques will be needed. At the present time, juvenile scallops, derived from my larval populations, are growing rapidly in a flowing laboratory sea water system at Woods Hole.

I am grateful to L. Harris of the University of New Hampshire for providing the opportunity to collect scallops at Isles of Shoals. Thanks are due R. Turner, R. Scheltema, P. Chanley and P. Boyle for critically reading the manuscript. P. Boyle also helped to prepare the illustrations.

SUMMARY

(1) Sexually mature *Placopecten magellanicus* from Isles of Shoals, New Hampshire, were observed to spawn in nature at 14°–16° C. In the laboratory, spawning occurred from 10°–15° C.

(2) Average sizes of developmental stages were: eggs, 64 microns diameter; swimming gastrula, 69 microns long by 63 microns diameter; earliest straight-hinge veliger, 105 × 82 microns, with a hinge line of 81 microns. The umbo stage began in larvae exceeding 175 × 155 microns. Pediveligers averaged 279 × 242 microns with a depth of 127 microns, and were inequivalved, the left valve being larger.

(3) Development from the zygote to the swimming gastrula took 30–40 hours at 12° C. The straight-hinge veliger stage was reached in four days at temperatures between 12° and 18° C.

(4) Veligers reared at 15° C reached the pediveliger stage in 28 days, and the first spat was observed on the 35th day. Veligers reared at 19° C suffered a mass mortality when approximately half grown.

(5) Larvae remained viable at salinities as low as 10.5‰, and exhibited normal swimming from 16.9‰ to 30.0‰ in a 42 hour test.

(6) Larvae showed a thigmotactic settling response to shell fragments, small pebbles, and glass fragments. Predominant settling was on the undersides of these objects. Pediveligers appeared to delay metamorphosis until suitable physical substrates for settlement were encountered.

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DIAPAUSE IN THE GEMMULES OF THE MARINE SPONGE, *HALICLONA LOOSANOFFI*, WITH A NOTE ON THE GEMMULES OF *HALICLONA OCULATA*[1]

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Haliclona loosanoffi is one of several marine sponges that produce special structures called gemmules (Hartman, 1958; Wells, Wells and Gray, 1964; Fell, 1974; and Simpson and Fell, 1974). Each gemmule consists of a mass of large granular cells enclosed within a collagenous capsule which may be fortified with spicules. In some cases such gemmules persist during certain unfavorable conditions and germinate, forming new sponges, when more favorable conditions recur. For example, in Southern New England, specimens of *Haliclona loosanoffi* degenerate during the late summer and early fall, leaving exposed on the substrate gemmules that were produced at the bases of the sponges. During the winter when the water temperature may fall to below 0° C and small protected bodies of water may be covered by ice for several weeks, this sponge occurs only in the form of gemmules. These gemmules germinate in late spring when the water temperature rises to about 20° C (Hartman, 1958; and Fell, 1974). On the other hand, at Hatteras Harbor, North Carolina *Haliclona loosanoffi* is abundant during the winter when the water temperature drops to at least 5° C. There this sponge is found exclusively in the form of gemmules during part of the warm summer (Wells *et al*, 1964).

In view of the apparent relationship of gemmule germination to water temperature, two types of studies were undertaken in order to examine this relationship in greater detail. First, a 3-year field study of *Haliclona loosanoffi* was made to determine with greater precision the times at which various events in the life history of this sponge occur. Secondly, the germination of gemmules at different temperatures in the laboratory was studied with the hope of gaining some information concerning the regulation of germination. These studies provide evidence for winter diapause in New England populations of *Haliclona loosanoffi*.

The occurrence of gemmules in a related species, *Haliclona oculata*, from Fishers Island Sound is also reported. Although many specimens of this sponge from the northeastern coast of the United States, including specimens from Long Island Sound, have been observed (see de Laubenfels, 1949; Hartman, 1958), there appears to be no previous record of gemmule production. However, Topsent (1888) has briefly described the gemmules of *Haliclona* (*Chalina*) *oculata* on the Channel Coast of France.

MATERIALS AND METHODS

Field studies

A 3-year study of *Haliclona loosanoffi* was conducted in the Mystic Estuary (Connecticut) in the region north of Mason Island. Observations and collections

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of specimens were made at approximately 2-week intervals during this period. The water temperature was also recorded. From March through December of the last 2 years water samples were taken for salinity determinations. The salinity of filtered samples (Whatman No. 1 filter paper) was measured to the nearest 0.5‰ with a Goldberg refractometer (American Optical).

The specimens were fixed in Bouin's solution in sea water, and each was dissected and examined under a dissecting microscope for the presence of gemmules and embryos (and/or large oocytes). Small samples of the specimens were then embedded in paraffin and sectioned serially at 10 μ. The mounted, deparaffinized sections were stained with hematoxylin and eosin. Examination of such histological sections revealed the stages of development of the gemmules and the presence of small oocytes and spermatic cysts.

Studies of *Haliclona oculata* in Fishers Island Sound (New York) were also made over a 3-year period. Specimens were collected from gravel bottom near the Dumpling Islands in about 30 to 40 feet of water, using 2-bushel oyster dredges. The specimens were preserved on board the boat immediately following their collection. The preservation and examination of the specimens was the same as for *Haliclona loosanoffi*.

Experimental studies

Gemmules of *Haliclona loosanoffi*, encrusting blades of eel grass and algae, were collected during the fall in the Mystic Estuary. Some of the gemmules were stored in the dark at 5° C in covered finger bowls containing sea water; and others were immediately prepared for culturing. The gemmules collected during 1970 were stored in Mystic Estuary sea water, while those collected during 1971 and 1972 were stored in Instant Ocean sea water (Aquarium Systems, Inc.). The Instant Ocean sea water with trace elements was made with glass-distilled water to produce a solution with a salinity of about 24‰. The sea water was changed in the storage vessels at approximately 2-week intervals.

Small pieces of eel grass (*ca* 4 × 3 mm) bearing clusters of gemmules were cultured on sheets of lens paper in covered 4 inch finger bowls containing about 150 ml of Instant Ocean sea water (24‰). Four to 12 such pieces of eel grass were cultured in a single bowl; and the sea water was usually changed at approximately 5-day intervals. In some cases the sea water was changed every other day once germination had begun. The cultures were maintained in the dark at a constant temperature (± 1° C) in B.O.D. incubators.

In most cases when the germination of gemmules under two different temperature regemés was to be compared, blades of eel grass covered by gemmules were cut into pieces across their width; and alternate pieces were put in one of 2 groups. One group of cultures was placed under one set of conditions, and the second group was put under another. In this way the 2 groups were as nearly identical as possible.

The germination of the clusters of gemmules was scored according to 5 arbitrary stages. The characteristics of each of these stages were as follows: stage 1—one or a few small masses of sponge tissue with no oscular tubes; stage 2—more extensive germination, but with less than half of the surface of the culture covered by sponge tissue, no oscular tubes; stage 3—half or more of the surface of the culture covered by sponge tissue with no oscular tubes; stage 4—less than half of the surface of the culture covered by sponge tissue, but with one or more oscular tubes; stage 5—half or more of the surface of the culture covered by sponge tissue with one or more oscular tubes. The average stage attained by any particular group of cultures is called the germination index (GI). A "P" is used after the germination index to indicate that germination was largely restricted to the edges of the cultures.

RESULTS

Field observations

In order to adequately evaluate experimental results, such as those presented below, detailed information about the occurrence of various events in the life history of *Haliclona loosanoffi* under natural conditions is needed. A population of this sponge was regularly sampled at approximately 2-week intervals for a period extending from March 1969 through February 1972. Other less regular observations were made both before and subsequent to this 3-year period. A preliminary report on this study has already been made (Fell, 1974). Here primary consideration is given to the gemmules; sexual reproduction will be the subject of another publication.

The present study was carried out in the Mystic Estuary, in an area that extends between Mason Island and Pequotsepos Brook. The water in this part of the esutary is shallow, ranging from about 2 to 4 feet in depth. The water temperatures during the winter and summer may differ by more than 25° C. During the months of June through September, the water temperature ranged from 19.0° C to 28.5° C (mean $23.0 \pm 2.5°$ C), while during the months of January and February, the water temperature was generally at or below 0° C (range $-2.0°$ C to 3.0° C, mean $-0.5°$ C). For a major portion of the cold period a layer of ice several inches thick covered the region. From March through May the water temperature rose steadily, and from October through December it sharply declined (see Table I). The salinity generally ranged betwen about 22‰ and 32‰ and was highest during the late summer and early fall (also see Pearcy, 1962).

Table I summarizes some of the observations made on the life history of *Haliclona loosanoffi*. The earliest time of year that active specimens of this sponge have been found in the Mystic Estuary is late May. Such specimens and some ungerminated gemmules were collected on 27 May 1969 and 1 June 1970. In May of 1969 and June of 1969 and 1970 a total of 42 specimens of *Haliclona loosanoffi* were found resting on the bottom. Nearly all of these specimens (39) enclosed empty gemmule capsules, and 9 of them were also associated with yet ungerminated gemmules. It is therefore evident that these specimens developed from gemmules. Pieces of eel grass bearing only ungerminated gemmules or gemmules in the initial stages of germination (Fig. 1) were also observed. The latter were found on 9 June 1969 and 1 June 1970.

Bottom specimens of *Haliclona loosanoffi* were not found until 15 July in 1971 and were not observed at all in 1972. However, such specimens were found on 12 June and 26 June 1973. The observed variation in the occurrence of specimens derived from gemmules is probably due in part to sampling problems and in part to differences in the production and survival of gemmules from year to year.

Apparently sexual reproduction is initiated soon after the sponges develop from gemmules. The 4 bottom-specimens of *Haliclona loosanoffi* collected in late May 1969 and most (34/38) of the bottom-specimens taken in June 1969 and 1970 possessed oocytes and/or embryos or spermatic cysts. Specimens with sexual reproductive elements were found through July.

Both bottom-specimens (many, at least, produced from gemmules) and specimens attached to living eel grass and algae (developed from sexually produced larvae) were found in the study area during most of the summer of some years (1969, 1970 and 1973). Gemmule formation was first observed in late June in some of the bottom-specimens and in late July in the sexually produced specimens attached to eel grass. Thus this process may begin within about one month after the germination of the gemmules produced during the preceding year. The production of gemmules was found to continue into October, the substrates of the sponges being progressively covered with gemmules as the specimens grow.

TABLE I

The occurrence of gemmules and active specimens of Haliclona loosanoffi *in the Mystic Estuary*

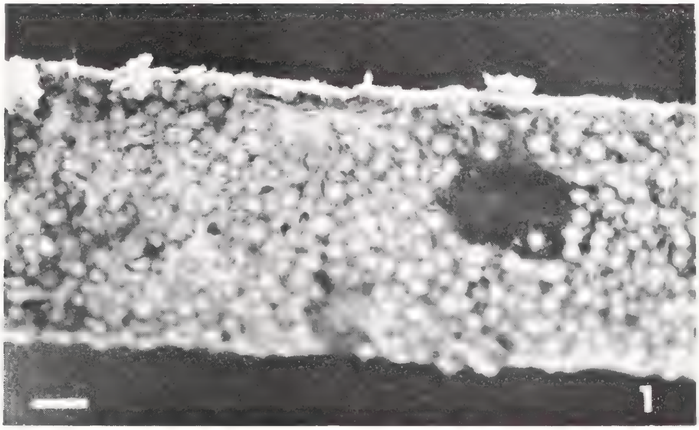
Month	Water temp. °C*	Active sponges	Gemmules			
			Developing	Formed	Exposed	Germinating
Jan.	−0.5	−	−	+	+	−
Feb.	0	−	−	+	+	−
March	5.5	−	−	+	+	−
April	10	−	−	+	+	−
May	16	+	−	+	+	+**
June	22	+	+	+	+	+
July	24	+	+	+	−	?
Aug.	24	+	+	+	±	−
Sept.	21	+	+	+	±	−
Oct.	16	±	+	+	+	−
Nov.	7.5	±	−	+	+	−
Dec.	2.5	−	−	+	+	−

* Mean surface water temperature at low tide.

** Not actually observed; but since some sponges have been found in May, some germination must occur during this month.

Regression began in some specimens of *Haliclona loosanoffi* by late August and early September; and some gemmules were exposed at this time. By early October there were many pieces of eel grass and algae bearing exposed gemmules. Many of these gemmules were completely exposed, while others were partially covered by a latticework of bare parental skeleton. Living specimens of *Haliclona loosanoffi* were associated with some of these gemmules, but not with many others. Many of the specimens showed evidence of degeneration. In some cases the sponge tissue was retracted slightly away from the basal gemmules, and in other cases it was separated from them by a broad zone of vacated skeleton (Fig. 2).

Furthermore, many of the specimens found at this time of year were a purplish color instead of the usual rosy beige or tan. The latest that "active" specimens of *Haliclona loosanoffi* were observed in the Mystic Estuary was mid-November. A few small lavender-colored specimens were found on 14 November 1970, but no active specimens were observed during this month in other years.



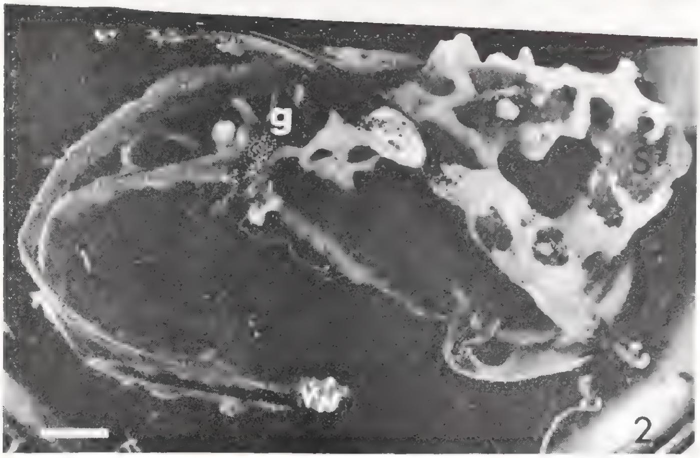


FIGURE 1. Living gemmules of *Haliclona loosanoffi* in an early stage of germination, collected on 9 June 1969 (scale bar = 1 mm).

FIGURE 2. Preserved specimen of *Haliclona loosanoffi* collected on 11 October 1969. Note the exposed gemmules (g) and vacated skeleton (s) (scale bar = 10 mm).

Gemmules were the only form in which *Haliclona loosanoffi* was found from November through late May. These gemmules encrusted pieces of dead eel grass and algae; and in some cases they were covered by the tissue of another sponge, *Halichondria bowerbanki,* which is sometimes abundant during the winter. The structure of the gemmules of *Haliclona loosanoffi* has been described by Hartman (1958) and Simpson and Fell (1974).

Germination of fall gemmules at 20° C

Gemmules collected on 4 October 1971 were used to prepare 50 cultures which were divided into two equal groups (A-1 and A-2). Both groups were initially placed at 20° C. After about one week most of the cultures showed germination of the gemmules along their edges, and many of the resulting sponges possessed oscular tubes (stage 4P). The germination index was 3.3P (see Fig. 3). After about 20 days many of the sponges showed signs of degeneration. The oscular tubes disappeared, and the sponge tissue began to gradually waste away. At this time all of the cultures still contained many ungerminated gemmules.

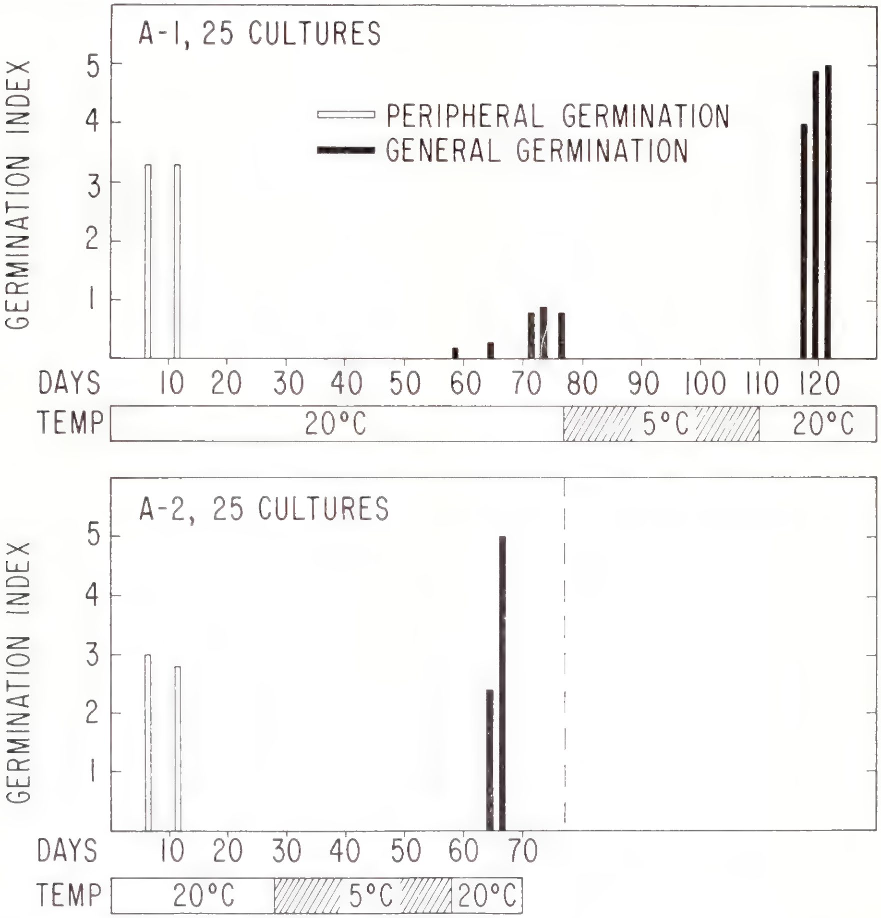


FIGURE 3. Germination of gemmules, collected on 4 October 1971, at 20° C before and after a 4-week period at 5° C.

The occurrence of germination along the edges of the cultures and especially along the cut edges was very striking (see Fig. 4). On the 9th day of culture 82% of the cut edges bore sponges compared with only 18% of the uncut edges and 22% of the upper surfaces. This pattern of germination suggests that physical damage to some of the cells of the gemmules and/or to the gemmule capsules may in some way stimulate the gemmules to germinate. This suggestion is strengthened by the fact that cultures of gemmules, which have no cut edges, do not exhibit such preferential germination at their periphery (see below).

On the 28th day one group of cultures (A-2) was placed at 5° C for 30 days. At the end of the cold treatment it was returned to 20° C. Seven days after the cultures were placed at the higher temperature, all of them had begun to germinate; and by the 9th day all of them had reached stage 5 (see Figures 3 and 5). Germination was therefore essentially complete on the 67th day of total culture. In the other group of cultures (A-1), which was kept at 20° C, the first indication of later germination was on day 59. Eventually 5 of the 25 cultures showed some germination (GI = 0.9), after which the sponge tissue began to regress. On day 77, which was 10 days after all of the A-2 cultures had reached stage 5, the A-1 cultures were placed at 5° C for 33 days. Then they were returned to 20° C. By the 12th day at 20° C (the 122nd day of total culture), all 25 cultures were at stage 5 (see Fig. 3).

In another experiment 17 cultures were prepared using large, uncut pieces of eel grass covered by gemmules (average size, *ca* 34 × 4 mm). The masses of gemmules, which were collected on 28 October 1971, were divided into 2 groups. One group of 9 cultures (B-1) was placed initially at 20° C; and the other group (B-2), which consisted of 8 cultures, was cultured at 5° C for one month before being placed at 20° C.

The B-1 cultures did not experience an early, peripheral germination. By day 29 only 2 cultures showed any signs of germination. Both of these cultures were at stage 1 (GI = 0.2). After 39 days the germination index was only 1.1; on day 46 it was 3.1; and on day 55 it was 3.7. In all of the cultures there were still many ungerminated gemmules. On the 55th day the cultures were placed at 5° C for 41 days. At the end of the cold treatment the cultures were returned to 20° C. By the 11th day at 20° C (the 107th day of total culture) all of the cultures had reached stage 5.

The B-2 cultures were transferred from 5° C to 20° C after 31 days of culture. By the 12th day at the higher temperature (the 43rd day of total culture) the germination index was 4.4. Five of the 8 cultures produced large sponges, but all of them still possessed many ungerminated gemmules.

The results of these experiments suggest that gemmules collected during the early fall do not readily germinate at 20° C and that low temperature enhances germination of the gemmules. Additional support for these conclusions is given by the experiment described in the next section.

Germination of fall gemmules at 20° C following a short period at 5° C

Four groups of cultures were set up using gemmules collected on 4 October 1971. These groups consisted of gemmules which had been stored at 5° C for different lengths of time (1, 2, 5 and 9 weeks) before being cultured at 20° C. All of the cultures derived from the same mass of gemmules were placed in the same finger bowl so that differences between different masses of gemmules (at least 3 per group) could be detected. Table II summarizes the results of this experiment.

Twenty eight cultures were set up using gemmules which had been kept at 5° C for one week. These were divided into 2 groups: group BA-1 containing 18 cultures and group BA-2 containing 10 cultures. When the cultures were placed at 20° C, they behaved like cultures which had received no cold treatment. After about one week there was extensive germination along the edges of the cultures (GI = 3.6P), but all of the cultures still contained many ungerminated gemmules. Again most of the germination occurred along the cut edges of the cultures. On the 7th day 87% of the cut edges were covered by small sponges, while only 29% of the uncut edges and 25% of the upper surfaces of the cultures bore sponges. Over a period of several weeks, the sponges in these cultures gradually wasted away.

On the 28th day the BA-1 cultures were placed at 5° C for 28 days and then returned to 20° C. By the 11th day at 20° C (the 67th day of total culture) most of the cultures exhibited extensive germination, and the germination index was 4.7. At this time the germination index of the BA-2 cultures, which had been kept continuously at 20° C, was only 0.8. On day 71 the BA-2 cultures were placed at 5° C for 31 days. At the end of the cold treatment the cultures were returned to 20° C, and by the 7th day at the higher temperature all of them had reached stage 5 (see Table II).

TABLE II

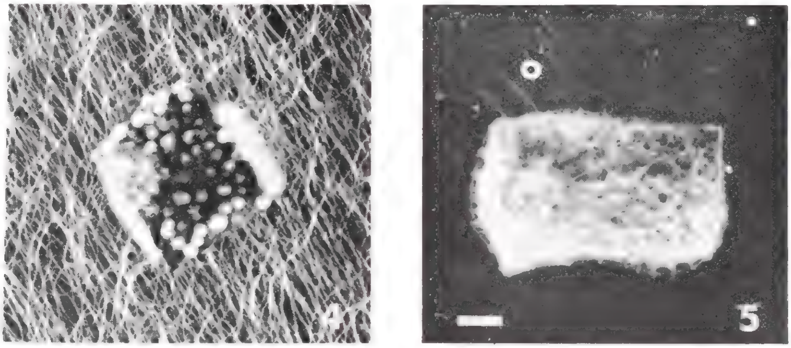
The effect of a period(s) at 5° C on the subsequent germination of gemmules at 20° C (all gemmules collected 4 October 1971); germination index: (A) initial germination at 20° C, (B) subsequent germination by end of temperature régime, (C) germination after a later 4-week cold treatment

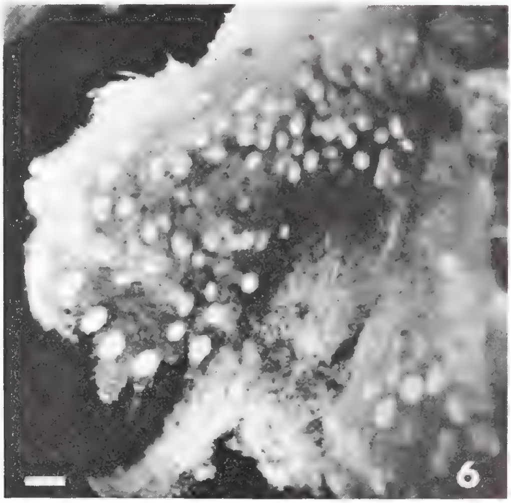
Exp.	No. cultures	Temperature régime*	Germination index		
			A	B	C
A-2	25	4-*4*-1	(3.3P)	5.0	—
A-1	25	10		0.9	5.0
BA-1	18	*1*-4-*4*-2	(3.6P)	4.7	—
BA-2	10	*1*-10		0.8	5.0
BB-1a	10	*2*-1	5.0	—	—
BB-1b	10	*2*-4-*3*-2	4.2 (3.5)	5.0	—
BB-2	12	*2*-9		1.3	5.0
BC	33	*5*-2	4.0	—	—
BD	30	*9*-2	4.9	—	—

* Number of weeks at 20° C in regular type; number of weeks at 5° C in italics.

A group of 8 cultures (BA-3) was prepared at the same time as the other two, using gemmules kept at 5° C for one week. The capsules of many of the gemmules in this group were pierced with a sharp iridectomy knife. After about a week at 20° C, 7 of the cultures showed extensive germination; and the germination index was 4.75. Admittedly the number of cultures is small, but the results lend further support to the suggestion that damage to the gemmules may cause early germination. Although many of the gemmules in this group of cultures germinated within 2 weeks, many others did not germinate until after they had received a 31-day cold treatment.

Thirty two cultures were prepared from gemmules which had been stored at 5° C for 2 weeks. These were divided into one group of 20 cultures (BB-1) and another group of 12 cultures (BB-2). Both groups of cultures were initially placed at 20° C. By the 8th day 14 cultures were at stage 5, and the germination index was 4.2. Ten of the stage-5 cultures (part of group BB-1) were from a single blade of eel grass. Excluding these cultures, the germination index was 3.5 or about the same as that for the cultures kept at 5° C for one week prior to being placed at 20° C. However, in this set of cultures there was little peripheral germination; only 10 of the 32 cultures had sponge tissue restricted to their periphery.





FIGURES 4 and 5. Cultures of gemmules collected on 4 October 1971: 4. 8-day culture at 20° C showing germination along the cut edges of the culture; 5. stage-5 culture on the 13th day at 20° C following a long period at 5° C. Note the oscular tube (o) (scale bar = 1 mm).

FIGURE 6. Surface of a specimen of *Haliclona oculata* that was formerly in contact with the substrate. Note the gemmules. Specimen was collected on 6 June 1972 (scale bar = 2 mm).

The 10 stage-5 cultures of group BB-1, which were from the same mass of gemmules, were discarded since most, if not all, of the gemmules had apparently germinated. After the remaining BB-1 cultures had been at 20° C for 30 days, they were placed at 5° C for 18 days. At the end of the cold treatment the cultures were transferred back to 20° C. By the 13th day at the higher temperature (the 61st day of total culture) all of the cultures were at stage 5. On the other hand, the BB-2 cultures, which had not had a second exposure to low temperature, had a germination index of only 1.3. On the 64th day of culture the BB-2 cultures were placed at 5° C for 31 days, and then they were transferred back to 20° C. After 9 days at 20° C all of these cultures had reached stage 5 (see Table II).

Gemmules, which had been stored at 5° C for 5 weeks, were used to set up 33 cultures. After about 2 weeks at 20° C the cumulative germination index was 4. Although in this case the germination index was somewhat low, in other experiments a 4 to 6 week period at 5° C was sufficient to produce nearly complete germination. This was true in experiments A, BA and BB (see Table II).

TABLE III

The germination of cold-treated gemmules at various temperatures (all gemmules collected in October)

Culture temp. °C	No. cultures	Weeks stored at 5° C	Days to max. germination	Germination index
10	34	43	—	0
15	42	30 & 39	avg. 22	5.0
20	180	4–9	avg. 11	4.73
20	146	30–41	avg. 10	4.96
25	35	41	12	5.0
30	69	31 & 35	avg. 10	3.96
34	18	38	—	0

Thirty cultures were set up using gemmules which had been stored at 5° C for 9 weeks. By the 11th day at 20° C nearly all of the cultures were at stage 5, and the germination index was 4.9. Although most of the cultures produced large sponges, there were still a number of ungerminated gemmules in many of the cultures.

Germination of fall gemmules at various temperatures after a long period at 5° C

Gemmules, which had been collected in October and stored at 5° C for from 30 to 43 weeks, were tested for their capacity to germinate at 10, 15, 20, 25, 30 and 34° C. In most experiments the germination of gemmules at 20° C was compared with that at some other temperature. The results of these experiments are summarized in Table III.

Thirty four cultures, which were kept at 10° C for 30 days, showed no evidence of germination. That the gemmules were healthy and capable of germination under favorable conditions was demonstrated by their response when they were transferred to 20° C. By the 9th day at the higher temperature all of the cultures had reached stage 5.

A total of 42 cultures were kept at 15° C. In one experiment involving 30 cultures, the first evidence of germination was on the 15th day and it was not until the 27th day that all of the cultures had reached stage 5. In another experiment, 12 cultures kept at 15° C all reached stage 5 by day 17. While the time required for germination was less than in the first experiment, it was substantially greater than that for 12 control cultures kept at 20° C. All of the latter cultures reached stage 5 on the 8th day of culture.

As has already been indicated, gemmules, which have had at least a 4-week cold treatment, germinate within about one to 2 weeks at 20° C. In a number of experiments involving over 300 cultures, the maximal germination index was usually achieved within 8 to 12 days, and 50% or more of the cultures showed evidence of germination within 5 to 7 days (see Table III).

Thirty five cultures kept at 25° C behaved essentially like cultures kept at 20° C, except that germination was accelerated by about 24 to 48 hrs. On the 5th day most of the cultures placed at 25° C were in early stages of germination, and one day later 28 of them were at stage 5. By comparison, only 4 of 35 control cultures kept at 20° C had begun to germinate by the 5th day, and none of them were at stage 5 on day 6. However, on the 7th and 8th days of culture the number of cultures to have reached stage 5 was 18 and 28 respectively.

A total of 69 cultures were placed at 30° C. Many of these cultures reached stage 5, but their development was inferior to that of gemmules kept at 20 or 25° C. In one experiment, involving 38 cultures, about half of the cultures reached stage 5; and the germination index was 3.7. In a second experiment, using 31 cultures, the germination index was 4.3. Cotnrol cultures (30) kept at 20° C were delayed in their germination by about 24 hrs compared to the cultures kept at 30° C, but all of them reached stage 5 by the 8th day of culture. Not only did more cultures reach stage 5 at 20° C, but the cultures appeared to be more robust. Twelve cultures of the second experiment, which did not reach stage 5 (GI = 3.2), were subsequently transferred to 20° C. After 9 days at the lower temperature the germination index of this group of cultures was 4.9.

Twenty nine cultures were placed at a variable high temperature (31.0 to 34.5° C). By the 8th day of culture only 10 cultures (all from one group of gemmules) showed any evidence of germination. On the other hand, 29 control cultures kept at 20° C had all reached stage 5 by this time. Already by the 7th day of culture sponge tissue, which resulted from the limited germination at high temperature, was beginning to regress. On the 8th day the remaining sponge tissue was removed from the gemmules with a sable brush, and the cultures were placed at 20° C. By the 13th day at 20° C (the 21st day of total culture) 27/29 cultures had attained stage 5 (GI = 4.8). This experiment suggests that the germination of the gemmules of *Haliclona loosanoffi* may be reversibly inhibited by high, as well as by low, temperature.

Finally, 18 cultures were kept at 34° C. These cultures showed no evidence of germination during 20 days at this temperature; and when they were subsequently transferred to 20° C, there was no germination during a 16-day period. By comparison most of the 18 control cultures placed at 20° C were in early stages of germination on the 6th day and had reached stage 5 by day 12 (GI = 4.9). This result suggests that a prolonged exposure of the gemmules to 34° C is lethal.

Additional observations

In a number of cultures it was noted that little or no germination occurred among the gemmules situated on the lower surface of the eel grass. However, when the cultures were recultured in an inverted position, there was frequently extensive germination among these gemmules. This observation suggests that conditions are less favorable for germination on the undersurfaces of the cultures where the gemmules are in contact with the substrate. This may be due to a restriction of respiratory gas exchange and/or an accumulation of certain metabolic byproducts.

It appears that the gemmules of *Haliclona loosanoffi* can be stored at 5° C under the conditions used in this study for only about one year. In all, 112 cultures were prepared from gemmules collected on 8 October 1970 and stored at 5° C for from 7 to 10 months. These cultures, which were kept at from 15 to 30° C, had a germination index of 4.7. The only cultures, which did not reach stage 5, were some of those kept at 30° C. On the other hand, 90 cultures derived from gemmules collected on the same date and stored at 5° C for from 16.5 to 19.5 months had a total germination index of only 0.1 when cultured at 20 or 25° C.

Finally, many of the gemmules, kept at 10 to 20° C for long periods of time without germination, became covered by a thin, dark brown layer of what appeared to be algal growth. However, this layer did not seem to significantly hinder the subsequent germination of the gemmules.

The gemmules of Haliclona oculata

More than 300 specimens of *Haliclona oculata* were dredged from Fishers Island Sound in the vicinity of the Dumpling Islands. Collections were made at irregular intervals over a period extending from May 1971 to February 1974 and during every month except September. In all of the 18 collections, many of the sponges (40 to 100%, avg. 76%) possessed gemmules. These were situated at the base of the stalk, either in contact with the substrate or only a few millimeters above it (Fig. 6). From March through June many of the specimens possessed oocytes, embroys, and/or larvae in progressively advanced stages of development. In some cases these occurred throughout the endosome from the fibrous stalk to the tip of the sponge. Some of the specimens, which lacked oocytes and embryos, were found to contain large numbers of spermatic cysts.

In many of the collections, a few of the specimens showed evidence of basal degeneration. In some cases the stalk was devoid of living tissue, but most of the rest of the specimen appeared to be healthy. In other cases the dead stalk supported only a few small masses of sponge. Dead stalks alone were also found. What appeared to be living gemmules were observed at the base of some of the dead stalks.

The number of gemmules produced by any specimen was relatively small. An exact enumeration of the gemmules was difficult, because they were frequently embedded in a dense network of spongin (collagen) fibers. However, it appeared that some of the specimens possessed fewer than 10 gemmules, while others contained as many as 70 or more. This is in general agreement with the observations of Topsent (1888); however, the estimate given by him that 30 is the maximal number of gemmules produced by a single specimen of this sponge is low. Although one specimen 57 mm in height was found to possess gemmules, it appears that these structures are generally restricted to larger specimens (> *ca* 80 mm in height). The gemmules were generally from about 600 μ to more than 1000 μ in their greatest diameter.

An attempt to bring about the germination of the gemmules of this sponge was unsuccessful. Fifteen groups of gemmules, collected on 4 May 1973, were placed in finger bowls in an aquarium (Dayno Aqua Lab) containing 20 gallons of freshly collected sea water (30‰) that was constantly circulated. One to 3 groups of gemmules were placed in each finger bowl. Four of the groups of gemmules were collected with dead stalks, and the rest were obtained from living specimens. The water in the aquarium was maintained at 8° C during the first 48 hours, after which it was allowed to come up to room temperature (*ca* 23° C). During 28 days of culture there was no evidence of germination, although the gemmules of *Haliclona loosanoffi* readily germinate within a few days under similar conditions (Olmstead and Fell, unpublished).

DISCUSSION

Haliclona loosanoffi and *Haliclona oculata* are among at least five species of *Haliclona* that are known to produce gemmules. The other species are *Haliclona* (*Chalina*) *gracilenta* (Topsent, 1888); *Haliclona ecbasis* (Fell, 1970); and *Haliclona permollis* (David Elvin, Oregon State, personal communication). However, not all haliclonids form gemmules. For example, in Long Island Sound *Haliclona canaliculata* overwinters in a simplified form lacking flagellated chambers, but does not produce gemmules (Hartman, 1958). Other gemmuliferous marine sponges include *Suberites domuncula* (Herlant-Meewis, 1948), *Suberites ficus* (Topsent, 1888; Hartman, 1958), *Prosuberites microsclerus* (Wells *et al*, 1964), *Laxosuberites lacustris* (Annandale, 1915), *Cliona vastifica* (Topsent, 1888; Annandale, 1915), and *Cliona truitti* (Wells *et al*, 1964).

A number of other sponges, including *Haliclona heterofibrosa* (Bergquist, Sinclair and Hogg, 1970), apparently produce gemmule-like masses which develop into free-swimming parenchymula larvae. Such "gemmules" usually are not in contact with the substrate of the sponge, and they do not possess a thick enveloping capsule. Although this form of asexual reproduction was first described by Wilson in 1891 and 1894, it remains poorly understood (see Fell, 1974).

The gemmules of *Haliclona loosanaffi* are frequently present throughout most of the year in the Mystic Estuary. They are produced beginning in June or July and do not germinate until the following May or June. During the late summer and early fall the parent sponges degenerate, leaving the gemmules attached to the substrate. The gemmules are the only form in which this sponge exists during the colder months of the year, and consequently at this location they are an obligatory part of the life history. Evidently the gemmules of this sponge are special structures which permit it to survive low temperature and other adverse conditions (also see Hartman, 1958).

In this study it has been shown that the gemmules of *Haliclona loosanoffi* may be stored at 5° C for up to 10 months without showing any signs of germination. However, such gemmules readily germinate when they are subsequently cultured at 20° C. Gemmules collected in early October have germinated in the laboratory as early as December and as late as September. Germination also occurs at 15° C but not at 10° C. Well developed sponges usually develop in about one to 2 weeks at 20° C or in approximately 2 to 4 weeks at 15° C. The results of these laboratory studies are in good agreement with field studies which suggest that germination normally occurs as the water temperature is rising from about 15 to 25° C. It therefore appears that germination of the gemmules is prevented during the winter by low temperature. A similar situation has been found to exist for the gemmules of several fresh-water sponges (Rasmont, 1954, 1962 and 1963; Strekal and McDiffett, 1974). However, the gemmules of *Spongilla lacustris* and *Trochospongilla* (*Tubella*) *pennsylvanica* (in Northern New England) normally germinate when the water temperature is still only 4 to 5° C (Simpson and Gilbert, 1973).

The regulation of gemmule germination in *Haliclona loosanoffi* is not as simple as it first appears. The gemmules begin to form early in the summer, and the completed gemmules become exposed on the substrate at the end of the summer when the water temperature may be close to 20° C or about the same as that occurring in the spring when the gemmules normally germinate. Some mechanism(s) must therefore be operating to prevent the gemmules from germinating prior to the onset of winter conditions.

Gemmules of *Haliclona loosanoffi*, collected in the early fall and cultured at 20° C without a period of storage at 5° C, do not germinate as readily as cold-treated gemmules. In a number of experiments, involving gemmules with either no or only a very short cold treatment, few of the gemmules germinated during approximately 2 months of culture at 20° C. On the other hand, cultures of gemmules, which had been kept at 5° C for about 4 weeks before being placed at 20° C, underwent extensive germination within a period of 2 weeks. These experiments suggest that some process(es) in the germination of the gemmules requires a relatively long period of time and that this process is accelerated by, but is not totally dependent upon low temperature. Such a condition is known as diapause (Agrell, 1951; Rasmont, 1954).

The diapause experienced by the gemmules of *Haliclona loosanoffi* appears to be very similar to that occurring in the gemmules of the fresh-water sponge, *Ephydatia mülleri*. Gemmules of the latter species, collected in July and maintained for nearly 4 months at 3.5° C, showed 90% germination after 8 days at 20° C, while gemmules, kept at 14 to 16° C for the same period, exhibited only 1% germination after 8 days and 11.5% germination after 20 days at 20° C (Rasmont, 1954). The timing of certain events in the life histories of these sponges is also similar. Near Brussels, Belgium *Ephydatia mülleri* reproduces sexually in May, produces gemmules primarily during June and July, and regresses, except for gemmules, by early fall (Rasmont, 1962). The gemmules of *Spongilla fragilis* and the brown gemmules of *Spongilla lacustris* also undergo diapause, but in these cases the diapause appears to be less deep (Rasmont, 1954 and 1955).

The exposure of the gemmules of *Haliclona loosanoffi* to 5° C for a period of one week has little effect on subsequent germination compared to that of unchilled gemmules. However, a cold treatment of only 2 weeks appears to result in a definite but variable enhancement of germination; and exposure to 5° C for 4 or more weeks usually leads to the germination of most of the gemmules when they are then cultured at 20° C. Similarily, Rasmont (1954) found that 3-day exposure to 3.5° C had no effect on the subsequent germination of the gemmules of *Ephydatia mülleri*, but that treatments of 12, 20 and 30 days resulted in progressively higher percentages of germination, 45%, 75% and 92% of the gemmules respectively.

The optimal temperature for breaking diapause in the gemmules of *Haliclona loosanoffi* has not yet been determined. However, it has been shown that 3° C and 8° C are equally effective in bringing about germination of the gemmules of *Ephydatia mülleri* (Rasmont, 1955). A temperature of 12° C was somewhat less effective than the lower temperatures but was substantially more effective than 18° C. Gemmules collected on 1 September and exposed to 3 or 8° C for one month subsequently showed 90% germination when they were cultured at 20° C. By comparison 60% of the gemmules exposed to 12° C and only 25% of those exposed to 18° C germinated when tested under the same conditions. However, it was found that after 2 to 3 months at 18° C, most of the gemmules were capable of germination. A similiar situation was shown to exist for the gemmules of *Spongilla fragilis*, but for this species 8° C was somewhat more effective than either 3 or 12° C in breaking diapause (Rasmont, 1955). If the gemmules of *Haliclona loosanoffi* are physiologically similar to those of these fresh-water sponges, 5° C should be close to the optimal temperature leading to the maturation of the gemmules.

When and how the diapause is initiated are unknown. One would like to know whether it is initiated when the gemmules are formed or at some later time. Also nothing is presently known concerning the nature of the diapause itself. However, one observation on the germination of gemmules, which had received no cold treatment or only a very short one, is of interest in this connection. Frequently many of the gemmules situated along the cut edges of the cultures germinated within a few days, while most of the rest of the gemmules did not germinate even after a long period of time. This suggests that injury to the gemmule capsule and/or to the cells of the gemmule may stimulate germination. Perhaps the gemmule capsule, like the coats of certain seeds, is important in maintaining dormancy either by restricting respiration or by retarding the loss and/or inactivation of germination inhibitors (Roberts, 1969).

Ephydatia fluviatilis and *Spongilla lacustris* produce an inhibitor of gemmule germination (so-called gemmulostasin), but *Ephydatia mülleri* does not (Rasmont, 1965; Rosenfeld, 1970). The first 2 species form gemmules late in the year and do not degenerate until the onset of winter conditions (Rasmont, 1962). The inhibitor produced by the parental tissue inhibits the germination of the gemmules until this time; and the gemmules of *Ephydatia fluviatilis* and the green gemmules of *Spongilla lacustris*, unlike those of *Ephydatia mülleri*, do not undergo diapause (Rasmont, 1954 and 1962). The inhibitors of *Ephydatia fluviatilis* and *Spongilla lacustris* are not species specific and inhibit the germination of the gemmules of *Ephydatia mülleri*. That of *Ephydatia fluviatilis* acts on an early phase of germination; and once this phase is past, the inhibitor has no effect on the later development of the new sponge. As would be expected, the effect of the inhibitor is reversible (Rosenfeld, 1970). It is of interest that if the capsule of the gemmules is pierced, germination is not inhibited by gemmulostasin although it is slowed compared to that of untreated controls (Rosenfeld, 1971). From the preceding discussion one would predict that *Haliclona loosanoffi* does not produce such an inhibitor of gemmule germination. Certainly if it does, the inhibitor could play only a minor regulatory role.

At Hatteras Harbor, North Carolina some specimens of *Haliclona loosanoffi* degenerate during the winter, but many others are present throughout this period when the water temperature falls to about 5° C (Wells *et al.,* 1964). Although the winter at Hatteras Harbor is apparently less severe than at Mystic, there presently seems to be no obvious explanation for the survival of *Haliclona loosanoffi* at the former location but not at the latter. In the Mystic Estuary the sponges degenerate in the late summer and early fall when the water is still warm. The gemmules exposed during the winter at Hatteras Harbor germinate in April when the water temperature reaches approximately 16° C, a situation similar to that in New England (Wells *et al.,* 1964).

On the other hand, at Hatteras Harbor *Haliclona loosanoffi* is absent, except for gemmules, during the middle of the summer when the water temperature is about 30° C. The summer gemmules, which germinate is September, may be of 2 types: those produced during the fall and winter and exposed to winter temperatures while enclosed by parental sponge tissue and those produced during the late spring and early summer (Wells *et al.,* 1964). It would be of interest to know whether these 2 classes of gemmules differ in their capacity to germinate. One class of gemmules was chilled and the other was not. However, the chilling of gemmules within the parental sponge may not have the same effect as that of chilling exposed gemmules.

The gemmules of *Haliclona oculata* appear to play a different biological role from that played by the gemmules of *Haliclona loosanoffi.* Specimens of the former sponge evidently remain active and gemmules apparently are present throughout the year. The gemmules may repopulate the substrate when specimens degenerate or when the sponges are torn loose during storms. Herlant-Meewis (1948) believes that the gemmules of *Suberites domuncula* play a similar role.

Although the present report is primarly concerned with the gemmules of *Haliclona loosanoffi* and *Haliclona oculata,* some information concerning sexual reproduction is given. In the Mystic Estuary specimens of *Haliclona loosanoffi* with reproductive elements are found in May, June and July (also see Fell, 1974). Larvae may be released as early as the middle of June, and the peak of larval settlement appears to occur in July (Fell, in preparation). The timing of reproduction is very different from that reported for this species at Milford, Connecticut (Hartman, 1958). At the latter location oocytes and embryos are present in specimens collected during late August and early September and the peak of larval settlement occurs during late September and early October. It is somewhat surprising that the reproductive periods of this sponge at 2 localities as near to each other as Mystic and Milford should be so different. At Hatteras Harbor, North Carolina *Haliclona loosanoffi* has 2 periods of larval settlement, one during June and July and the other in October and November (Wells *et al.,* 1964).

At Mystic, Connecticut and Hatteras Harbor, North Carolina (Wells *et al.,* 1964) sexual reproduction is initiated very soon after sponges develop from gemmule germination. A similar situation has been found to exist for the freshwater sponges, *Spongilla lacustris* and *Trochospongilla (Tubella) pennsylvanica* (Simpson and Gilbert, 1973). Furthermore, in *Spongilla lacustris* oocytes begin to develop within one week after refrigerator-stored gemmules are inplanted back into the natural habitat of the sponge, even when this is done well after the normal period of sexual reproduction (Gilbert, 1974). These facts suggest that there may be a regulatory connection between sexual reproduction and gemmule germination. However, gemmulation does not appear to be an obligatory prerequisite to sexual reproduction in these species. Larva-derived specimens of *Spongilla lacustris* (Simpson and Gilbert, 1974) and *Haliclona loosanoffi* (Fell, in preparation) may exhibit low levels of gamete production.

It is reported here that the reproductive period of *Haliclona oculata* in Fishers Island Sound extends from March through June. The only other reference to the reproductive period of this sponge is that of Hartman (1958). He reported the occurrence of reproductive specimens in Block Island Sound during July.

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SUMMARY

1. The gemmules of *Haliclona loosanoffi* are present throughout the year in the Mystic Estuary, but active specimens of this sponge are found only during the period extending from late May to late October or early November. Germination of the gemmules occurs primarily during May and June, and new gemmules are produced from late June through early October. The gemmules of this species evidently are a means for surviving adverse environmental conditions.

2. Both gemmules and active specimens of *Haliclona oculata* are found throughout the year in Fishers Island Sound. The gemmules of this species may repopulate the substrate when the parent sponges degenerate or are ripped loose during storms.

3. Gemmules of *Haliclona loosanoffi* collected in the fall do not germinate readily when they are cultured at 20° C. There is frequently only limited germination after 1 to 2 months at this temperature. However, if the gemmules are first put at 5° C for 4 or more weeks, they usually germinate within a few days after being placed at 20° C. Thus low temperature appears to enhance germination of these gemmules which undergo diapause.

4. Although low temperature appears to accelerate maturation of the gemmules of *Haliclona loosanoffi*, it also inhibits actual germination. Gemmules do not germinate at either 5 or 10° C. However, germination occurs within 2 to 4 weeks at 15° C and within 1 to 2 weeks at 20° C. At 25° C germination appears to be accelerated by approximately 24 to 48 hours compared to that occurring at 20° C.

5. The gemmules of *Haliclona loosanoffi* may be stored in the dark at 5° C for at least 10 months without any detectable reduction in their capacity to germi-

nate at higher temperatures. However, such gemmules can not be stored indefinately. Gemmules kept at 5° C for from 16.5 to 19.5 months did not germinate when they were subsequently placed at either 20 or 25° C.

6. Gemmules of *Haliclona loosanoffi* germinate at 30° C, but this temperature appears to be less favorable than 20 or 25° C. Slightly higher temperatures inhibit germination, and continuous exposure to 34° C apparently is lethal.

7. The reproductive period of *Haliclona loosanoffi* in the Mystic Estuary extends from late May through July and that of *Haliclona oculata* in Fishers Island Sound extends from March through June.

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HISTOCHEMICAL OBSERVATIONS ON THE LOCALIZATION OF SOME ENZYMES ASSOCIATED WITH DIGESTION IN FOUR SPECIES OF BRAZILIAN NEMERTEANS

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The processes of digestion have been investigated histochemically for several nemertean species (Jennings, 1962a; Gibson and Jennings, 1969; Jennings and Gibson, 1969; Gibson, 1970), and results so far obtained suggest that although the fundamental digestive sequence is essentially similar for all the species, differences in details can be related either to their systematic position or mode of life.

Irrespective of the nature of the food utilized, digestion occurs in two distinct phases. Initial extracellular digestion is accomplished in the intestinal lumen at an acidic pH and involves mainly proteolytic enzymes secreted by the gastrodermis. Food particles are subsequently engulfed by lamellar outgrowths of the distal ciliated cell walls (Jennings, 1969) and food vacuoles passed back into the gastrodermis for the second, intracellular, stage in digestion. This involves exopeptidases (arylamidases), acid and alkaline phosphatases and, in some species at least, carbohydrases and lipases.

The most uniform patterns of digestion are found in anoplan nemerteans (Jennings, 1962a; Jennings and Gibson, 1969). Acidophilic gland cells in the foregut, rich in carbonic anhydrase, discharge their contents during ingestion which serve both to kill prey taken alive and provide the correct lumenar pH for extracellular digestion. Mucoid secretions, discharged from other foregut glands, lubricate the food as it is passed into the intestine. Food entering the intestinal lumen stimulates the gastrodermal gland cells to discharge endopeptidases, which are responsible for early proteolysis and function optimally under acidic conditions.

Intracellular digestion initially involves endopeptidases phagocytosed along with food particles and is marked by a sharp increase in acid phosphatase activity in and around food vacuoles. Acid phosphatases may be concerned in some way with the maintenance of the correct pH for intralumenar endopeptic activity (Jennings and Gibson, 1969) or with food vacuole formation (Rosenbaum and Rolon, 1960), Slinger and Gibson (1974) demonstrating biochemically that these enzymes function optimally in the range pH 4.1–5.0, depending upon the species.

A few hours after the commencement of phagocytosis acid phosphatase and endopeptidase activity in the food vacuoles and surrounding cytoplasm declines, being replaced by alkaline phosphatases and exopeptidases. This final, alkaline, phase in digestion operates within the pH range 8.7–10.1 (Slinger and Gibson, 1974), and continues until digestion has been completed.

Endopeptidase enzymes can be demonstrated in the gastrodermal gland cells at all times, irrespective of the nutritive state, but exopeptidase activity can only be visualized histochemically in gut cells at the appropriate stage in digestion.

This contrasts markedly with blood system exopeptidases, which can be detected at all times (Gibson and Jennings, 1967).

Far greater variation in digestive physiology is found amongst the Enopla. At least two distinct types of foregut physiology have been demonstrated, one resembling the anoplan pattern and involving carbonic anhydrase production (*Prostoma*), the other achieving an intralumenar acidic pH via some other mechanism and not possessing demonstrable carbonic anhydrase (*Amphiporus, Paranemertes, Tetrastemma*) (Jennings and Gibson, 1969; Gibson, 1970).

Differences between the two classes are also found in the gastrodermal physiology; no enoplan species has yet been recorded with endopeptidase enzymes located in its gastrodermal gland cells, the enzymes instead being synthesized in and secreted from spherical inclusions housed within the columnar cells. The nature of the enzymes secreted by hoplonemertean gastrodermal glands has not yet been determined, but it is supposed that since the animals are carnivorous the secretions are proteolytic in form. Other enzymes involved in digestion essentially follow the pattern outlined for the Anopla.

A major departure from the usual type of digestive physiology is found in the bdellonemertean *Malacobdella* (Gibson and Jennings, 1969), where both morphological and physiological characters of the gut are considerably altered. These modifications, however, such as the replacement of endopeptidases by α-amylase-like carbohydrases as the principal enzymic group in digestion, and the total absence from the gastrodermis of demonstrable exopeptidases, can be entirely related to the species' atypical way of life and unselective microphagous feeding habits.

In the present study species of nemerteans belonging to families not previously investigated have been examined to determine whether or not existing known patterns of digestive physiology are evident, or whether additional variations could be detected.

MATERIALS AND METHODS

The species of nemerteans investigated, listed systematically, were:

ANOPLA

Order: HETERONEMERTEA

Baseodiscus delineatus (Delle Chiaje)

ENOPLA

Order: HOPLONEMERTEA

Ototyphlonemertes affinis Kirsteuer, Ms. name
Ototyphlonemertes erneba Corrêa
Ototyphlonemertes lactea Corrêa

Ototyphlonemertes affinis is soon to be described as a new species by Dr. Ernst Kirsteuer, The American Museum of Natural History, New York (Morphology, taxonomy and ecology of the nemertean genus *Ototyphlonemertes,* with special reference to the American species—in preparation).

Specimens of *Baseodiscus* were collected from beneath stones and boulders in the intertidal zones of shores at Ubatuba, São Sebastião and Praia de Siriuba, on the coast of São Paulo State, Brazil. The three *Ototyphlonemertes* species, all psammobiontic or interstitial forms (Kirsteuer, 1967, 1971), were obtained from a sandy beach at Ilhabela on the Island of São Sebastião, some 100 km east of Santos, Brazil. Samples of sand were "panned" in the manner employed by Corrêa (1958), fresh fish meat being used to bait the sand surface for about ten minutes before panning was carried out.

The living worms were maintained in the laboratory in frequently changed, cool sea water. A selection of associated fauna and artificial foods was tested in attempts to set up a feeding series, but under laboratory conditions none of the species were ever observed to feed. Results, therefore, are based upon histological and histochemical evidence obtained from different animals fixed at progressive time intervals after collection. In the case of the *Ototyphlonemertes* species, although responding to the first bait and often showing signs of having fed on it, a definite feeding series could not be established because there was no way of knowing when the animals' last meal had been prior to accepting the bait.

Histological observations were made on paraffin wax (56° C mp) sections of specimens fixed in marine Bouin and stained either by Mallory's trichrome or the 1% aqueous Alcian blue methods.

The nature and location of enzymes was investigated in animals fixed for 2–4 hr at 4° C in 10% buffered formalin, pH 7.0, washed in chilled distilled water and frozen-sectioned at 10–12 μ on an International Equipment Co. Microtome-Cryostat Model CTF. Sections were air-dried on clean slides and rinsed in cold absolute acetone before incubation for enzyme visualization.

The following methods were used to investigate enzymes present: the Hausler (1958) technique for carbonic anhydrase; the indoxyl acetate (Holt, 1958) and α-naphthyl acetate (Gomori, 1952) methods for non-specific esterases; the Burstone and Folk (1956) L-leucyl-β-naphthylamide method for exopeptidases (arylamidases); the Burstone (1958) azo-dye method for acid phosphatases, with naphthyl AS-TR phosphate as substrate and Red-violet LB salt as simultaneous coupler; the Gomori (1952) Tween method for lipases; and the Gomori (1939) calcium salt method for alkaline phosphatases, with sodium β-glycerophosphate as substrate.

Controls for these histochemical methods included the use of heat inactivated sections and incubation media from which the specific substrates were omitted.

In addition, fat deposits were studied in paraffin wax sections of animals fixed in Flemming's osmium tetroxide fluid, and the occurrence of glycogen reserves investigated in sections after fixation in 90% alcohol containing 1% picric acid and stained by the Best's carmine method.

OBSERVATIONS

ANOPLA

Order: HETERONEMERTEA

Baseodiscus delineatus

Structure of the gut. The gut is divisible into two principal regions, the foregut and intestine, both of which are lined by an epithelium formed from glandular and ciliated columnar cells. By far the shorter of the two alimentary regions, the foregut can be differentiated from the intestine by its mucus-secreting gland cells and shorter, more densely arranged, cilia.

The mouth, only 1 mm or less in length, opens into a buccal cavity with walls which are deeply folded into longitudinal ridges. These ridges have been reported from other *Baseodiscus* and heteronemertean species and are interpreted as permitting the dilation of the buccal cavity for the ingestion of a large-sized meal (Jennings and Gibson, 1969; Gibson, 1974). The buccal and foregut epithelium are histologically identical but differ in their thickness. Where buccal folding is most prominent the epithelium may be up to 300–350 μ tall whereas further back in the foregut it decreases to a height of only 200–250 μ. Cilia of the columnar cells in both buccal cavity and foregut are densely arranged and 6–8 μ long.

There appear to be several types of gland cells in the anterior gut epithelium, although two major varieties can be distinguished. These are: (1) Elongate or pyriform acidophilic glands filled with numerous closely packed minute spheres 1 μ or less in diameter. Other, similarly shaped, glands with more loosely arranged contents and less obvious acidophilic affinities, may represent gland cells of the same type but in a different physiological state. The glands are negative to Alcian blue; (2) Irregularly-shaped gland cells filled with a coarsely granular cytoplasm, approximately twice as numerous as the acidophils. Many of these glands stain with the Alcian blue method for mucopolysaccharides, the intensity of staining varying from faint to deep.

Large numbers of gland cells of both groups can also be found in the parenchyma underlying the foregut and buccal epithelium, discharging their contents through the gut wall into the lumen. A similar situation has been found in lineid heteronemerteans (Jennings, 1960, 1962a; Jennings and Gibson, 1969).

The intestine is typically anoplan in form and for most of its length bears serially repeated lateral diverticula. The intestinal epithelium, or gastrodermis, is identical in both the main canal and the lateral diverticula. It consists of ciliated columnar cells, up to 150 μ tall and 6–8 μ wide and with sparsely distributed cilia 10–12 μ long, interspersed with rather smaller pyriform gland cells containing oval or spherical acidophilic globules which show a strong positive reaction to methods for the demonstration of esterases (Fig. 1). The gland cells lie proximally in the gastrodermis, tracts of discharging globules reaching up to the intestinal lumen between the columnar cells.

Towards the posterior of the body there is a gradual reduction both in the size and number of lateral diverticula and in the gastrodermal height. Immediately before the anus the columnar cells are no more than about 50–60 μ tall.

The density of gastrodermal gland cells similarly decreases posteriorly and they are completely absent from the short "rectal" region, 3.5 mm long in an animal 27 cm in length. The anus opens at the extreme posterior tip of the body.

Enzymes of the gut. Carbonic anhydrase activity has been previously reported from the foregut acidophil glands of both palaeo- and heteronemerteans (Jennings, 1962a; Jennings and Gibson, 1969), where it is believed to be concerned in the initiation of the correct intralumenar pH for the subsequent extracellular phases of proteolysis. No evidence of carbonic anhydrase activity could be demonstrated in the foregut or any other tissue in *Baseodiscus delineatus.*

The Hausler method does, however, stain many elongate ovoid bodies located in the epidermis. These bodies, which are equally strongly stained in heat-denatured control sections, are of similar size and shape to the epidermal rhabdite-like cells and the reaction in them may be due to some calcium or other salt component.

Intense esterase activity, demonstrable with both the α-naphthyl acetate and indoxyl acetate techniques, was consistently found in some 10–12% of the buccal and foregut acidophilic gland cells (Fig. 2). Esterases have not previously been recorded from these sites in any nemertean species.

In the intestine the pyriform gastrodermal glands at all times stain strongly for esterase activity (Fig. 1). Although it was not possible to utilize selective inhibitors and activators in conjunction with the indoxyl acetate method, as employed by Hess and Pearse (1958), it seems likely that the gland cell activity represents that of cathepsin C-type proteases, as recorded from other anoplan forms (Jennings, 1962a; Jennings and Gibson, 1969). There is no reason to suppose that intestinal endopeptidases are absent from this species, and both histochemical methods employed are known to react positively at sites of endopeptic activity in other nemerteans.

Sections of animals prepared eight or more hours after collection showed that numerous food vacuoles and their surrounding columnar cell cytoplasm stained positively for esterase activity. The intensity of the colored reaction product varied between specimens; in some examples only a weak activity could be seen, confined mainly to the proximal regions of the columnar cells, but in others the activity was distributed throughout the gastrodermis and appeared very much stronger.

Arylamidases (exopeptidases) of the "leucine aminopeptidase" type were visualized in vacuolar and cytoplasmic regions of the gastrodermal columnar cells in only some of the specimens investigated (Fig. 3). When present the reaction, in general, appeared to be more intense in the posterior half of the intestine. Maximum arylamidase activity in food vacuoles occurred in animals with a weak vacuolar esterase reaction. Nemertean gastrodermal arylamidases are known to be histochemically demonstrable only during the appropriate phase in the digestive sequence and recent work by Slinger (1974) has shown that whole-animal arylamidase extracts function optimally on the alkaline side of neutrality.

In the gut acid phosphatase activity was confined to the intestinal columnar cells, in and around food vacuoles (Fig. 4). Its degree of activity and intracellular distribution closely parallelled that shown by the gastrodermal esterases.

Alkaline phosphatase activity in the intestine (Fig. 5) is similar to that of arylamidases, its maximum visualization in cytoplasm and food vacuoles occurring at the same time. The link between these two enzyme groups has already been clearly established in nemertean worms (Jennings, 1962a; Jennings and Gibson, 1969; Gibson, 1970), and both succeed esterase and acid phosphatase demonstration in the digestive sequence.

At times when arylamidase activity can not be shown in the gut, alkaline phosphatases are confined to the distal border of the gastrodermis, forming a narrow but intensely staining zone of activity a few microns deep. The occurrence of alkaline phosphatases at this site, where they are believed to be independent of the nutritive state, has been found in other heteronemertean species (Jennings, 1962a; Jennings and Gibson, 1969).

The Tween method for the demonstration of lipases has not generally proved to be sensitive enough with nemertean tissues, unless the animals are maintained on a high fat content diet (Jennings, 1962a). In some of the *Baseodiscus* specimens studied a faint but definite brownish reaction, absent from control slides, could with prolonged incubation be distinguished in several food vacuoles. This activity, occurring at the same time as peak arylamidase visualization, may represent the occurrence of lipolytic enzymes.

No other enzymes were demonstrated in any part of the alimentary system.

Food reserves. Fat deposits are principally restricted to the distal half of the gastrodermal columnar cells, occurring as droplets of varying diameter from 5–6 μ downwards. Glycogen, appearing as small granules scattered throughout the gastrodermis, can also be found in lesser quantities in freshly collected animals.

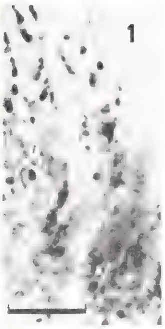
Enzymes of other tissues. Several enzymes were demonstrated at sites of activity other than the gut.

Arylamidase activity was consistently found in the blood system endothelium, although the intensity of the staining reaction varied from weak to strongly positive depending upon the part of the body and individual animal. The reaction was generally strongest in the lateral blood vessels where they ran alongside the intestine. Blood system arylamidases are well known from nemerteans (Gibson and Jennings, 1967).

Esterase activity was localized in many tissues of the body, including the epidermis (Fig. 6), ciliated cerebral canal and some parts of the body wall and proboscis musculature. At these sites the degree of activity was normally weak to moderate, although the proximal epidermal regions were often intensely stained. Consistently strong esterase activity was found in ganglionic cells of the principal nerve tracts, especially around the fibrous core of the cerebral ganglia and lateral nerve cords (Figs. 6 and 7). No instance was observed of any esterase or other enzymic activity in the fibrillar component of the nervous system, and it is probable that the esterases demonstrated are in fact of the cholinesterase type, as found in the nervous system of other nemertean species (Ling, 1969a, 1969b).

Esterase activity could, in some examples, also be localized in certain gland cells of the proboscis epithelium.

Alkaline phosphatases were irregularly found at several sites. Strong activity was seen in the nephridial ducts and cerebral canal, weak to moderate activity showed in the gland cell region of the cerebral organs. Other cells of the cerebral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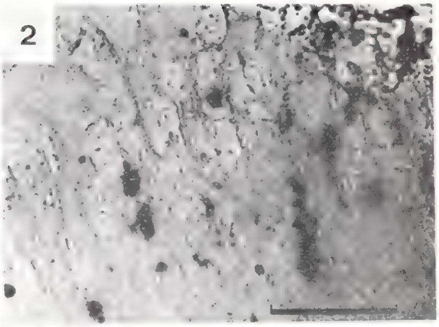
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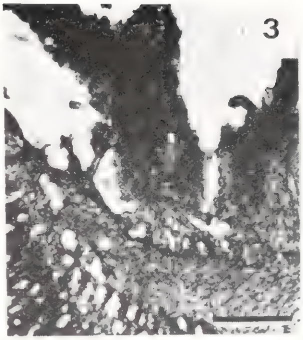
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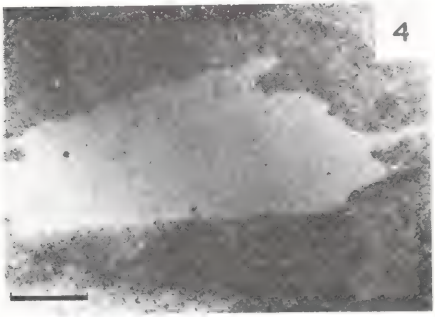
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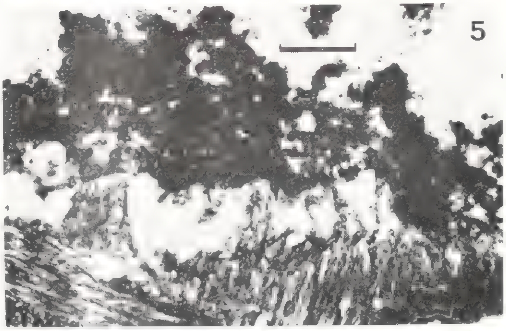
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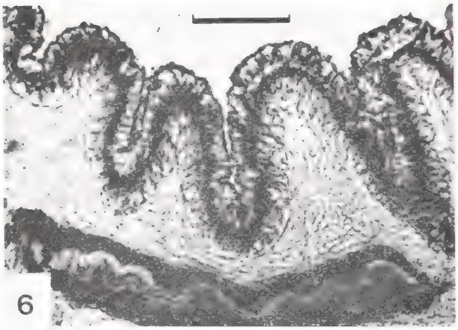
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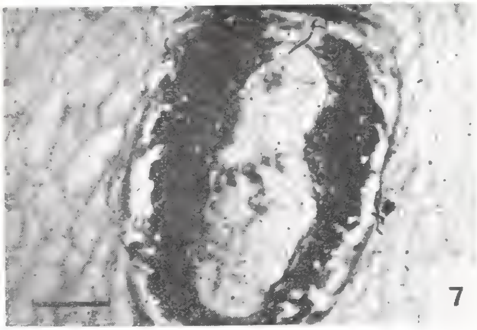
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FIGURE 1. *Baseodiscus delineatus;* Longitudinal section through part of the gastrodermis to show acidophilic gland cells staining intensely with the Holt indoxyl acetate method for esterases (proteases), scale = 105 μ.

FIGURE 2. *Baseodiscus delineatus;* Longitudinal section through a portion of the foregut epithelium to show esterase activity localized in some of the acidophilic gland cells; Gomori α-naphthyl acetate method, scale = 70 μ.

FIGURE 3. *Baseodiscus delineatus;* Transverse section through a part of the gastrodermis, showing arylamidase activity (black) generally distributed throughout most of the columnar cells; Burstone and Folk method, scale = 150 μ.

FIGURE 4. *Baseodiscus delineatus;* A part of the intestine showing acid phosphatase activity distributed through the distal regions of the gastrodermis; Burstone azo-dye method, scale = 130 μ.

organs on occasion reacted faintly to the acid phosphatase technique. Traces of these enzymes were also found in the proximal regions of the epidermis, but the reaction was always extremely weak and, although absent from control slides, not positively identified.

No other sites of enzymic activity were recorded in *Baseodiscus delineatus.*

ENOPLA

Order: HOPLONEMERTEA

Ototyphlonemertes affinis, O. erneba and *O. lactea*

Structure of the gut. The gut structure of two of the three species has been described by Corrêa (1950, 1954) and all are extremely similar. In common with other monostiliferous hoplonemerteans, the rhynchocoel and buccal cavity of *Ototyphlonemertes* share a common anterior aperture, the rhynchodaeal pore. This opens into the rhynchodaeum from which the rhynchocoel leads dorsally and the oesophagus ventrally.

Three regions of the gut can be recognized histologically, the oesophagus, the stomach and intestine. In the Brazilian *Ototyphlonemertes* the intestine lacks a caecum, the stomach therefore opening directly into it without the intervention of a pyloric tube such as is found in many hoplonemerteans.

The oesophagus opens from the rhynchodaeum in front of the cerebral ganglia and extends posteriorly below the brain as a short straight ciliated tube devoid of gland cells. It opens directly into the stomach, which consists of a folded epithelium formed from densely ciliated columnar or cuboidal and large numbers of gland cells. The epithelial height differs between the species; in *O. lactea* the stomach lining is only 10–12 μ tall compared with 25 μ in *O. affinis* and 30–35 μ in *O. erneba.* Most of the gland cells contain a finely granular to fibrillar basophilic secretion, but isolated acidophilic glands filled with homogeneous contents are irregularly distributed between the basophils. Stomach columnar cell cilia in all three species are densely arranged and 4–4.5 μ long.

Posteriorly the stomach opens directly into the intestine with only a slight narrowing of its lumen, unlike the situation depicted for *O. lactea* by Corrêa (1954; Plate 7, fig. 30).

The gastrodermis is very similar in structure to that described for other hoplonemerteans, consisting of acidophilic gland cells interspersed with sparsely ciliated columnar cells. The distribution of gland cells is more or less uniform throughout the intestinal length in *O. affinis* and *O. erneba,* but in *O. lactea* the anterior half contains many more gland cells than the posterior, the ratio being approximately 3 : 1. Corrêa (1954) notes the anterior aggregation of gland cells

FIGURE 5. *Baseodiscus delineatus;* Longitudinal section through part of the intestine to show the cytoplasmic localization of alkaline phosphatase activity; Gomori calcium salt method, scale = 83 μ.

FIGURE 6. *Baseodiscus delineatus;* Longitudinal section to show intense esterase activity present in the proximal epidermal regions and ganglionic cells of a lateral nerve cord; Gomori α-naphthyl acetate method, scale = 270 μ.

FIGURE 7. *Baseodiscus delineatus;* Transverse section through a lateral nerve cord, showing esterase activity confined to the ganglionic nerve sheath; Holt indoxyl acetate method, scale = 67 μ.

in *O. lactea,* and in this and other species calls them erythrophil glandular cells. The glands are filled with acidophilic spheres 1 μ or less in diameter, similar sized or slightly larger globules also occurring in the columnar cells, particularly in the proximal half. Columnar cell inclusions are similar in appearance to those described from other hoplonemerteans (Jennings and Gibson, 1969; Gibson, 1970) which contain endopeptidases. The cilia of the gastrodermis are 7–8 μ long, the overall gastrodermal height some 45–50 μ in all three species. Corrêa (1948) contrasts the low epithelial height of the stomach with the high gastrodermal development in other species (*O. brevis* and *O. evelinae*) without mentioning cellular dimensions. In the animals used during the present investigation the contrast between stomach and intestinal height depends upon the species concerned and is least in *O. erneba.*

As Corrêa (1950, 1954) reported, the "rectal" region of the intestine is not dilated and can be distinguished from the more anterior intestinal regions by the absence of gland cells. The region also lacks diverticula, but throughout the intestinal length these are at best only poorly developed.

Enzymes of the gut. No evidence of carbonic anhydrase activity could be found in the gut or any other tissue of the body.

Strong esterase activity was found in the gastrodermal acidophilic glands (Fig. 8), demonstrable by both histochemical methods employed. The individual globules filling the glands stained intensely in most of the animals studied, but in others could not be distinguished. Jennings (1962a) found that in the heteronemertean *Lineus ruber* gastrodermal gland cells which had discharged their spheres shortly after feeding failed to react to techniques for the visualization of cathepsin C-type endopeptidases. It thus seems probable that in those *Ototyphlonemertes* specimens with negative gland cell reactions a similar situation is prevailing, an inference supported by the fact that this was only seen in animals fixed soon after collection.

No other hoplonemertean species yet investigated has possessed histochemically demonstrable enzymes in its gastrodermal gland cells (Jennings and Gibson,

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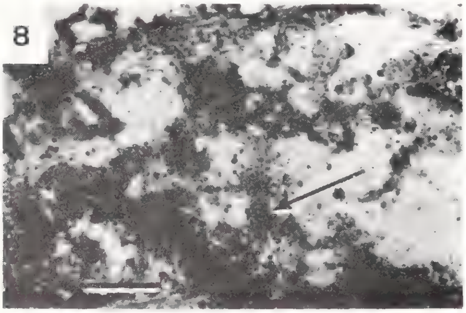

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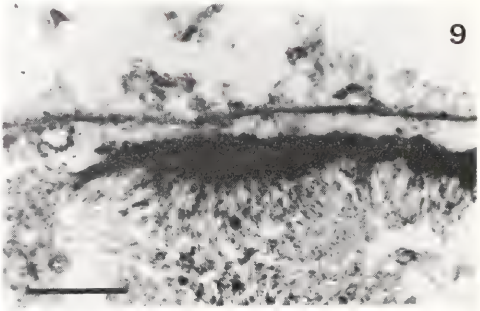

FIGURE 8. *Ototyphlonemertes affinis;* Longitudinal section through the gastrodermis to show the occurrence of esterases (proteases) in food vacuoles and gland cells. A pair of large gland cells is arrowed; Holt indoxyl acetate method, scale = 42 μ.

FIGURE 9. *Ototyphlonemertes erneba;* Longitudinal section through the anterior proboscis to show the localization of esterase activity in the basal regions of the epithelium. Some activity can also be seen in more distal parts of gland cells; Gomori α-naphthyl acetate method, scale = 67 μ.

1969; Gibson, 1970); instead endopeptidases are produced by and secreted from acidophilic globules contained in the columnar cells. In *Ototyphlonemertes* gastrodermal cytoplasmic inclusions do react positively for esterases but, in the absence of a feeding series, it cannot with certainty be determined whether these represent food vacuoles or the more usual hoplonemertean endopeptic vesicles. However, Corrêa (1950; page 218), in her histological study of the digestive processes in *O. brevis*, makes no mention of columnar cell inclusions other than after extracellularly digested material has been "absorbed" and shows as ". . . droplets, granules, and vacuoles of different size . . ." This evidence, together with the fact that esterase positive vesicles are not always visible, and that when they are there is also evidence of cytoplasmic activity around them, leads us to the conclusion that in *Ototyphlonemertes* the inclusions represent food vacuoles undergoing intracellular digestion. No other esterase activity could be distinguished in the digestive tract.

Little evidence of arylamidase activity could be found in the gut. A faint reaction, absent from control slides, appeared in the gastrodermal tissues of *O. lactea* and *O. erneba*, none being discerned in *O. affinis*. In specimens exhibiting this faint activity food vacuoles were negatively responsive to the esterase methods and, conversely, when strong vacuolar esterase activity was seen no evidence of arylamidases could be distinguished.

Acid phosphatase activity in the gut was entirely confined to gastrodermal food vacuoles and their surrounding cytoplasm, appearing most intense at the same time as maximal vacuolar esterase activity. When evidence of intracellular esterase function was missing, no acid phosphatases could be demonstrated in the gut.

Varied reactions to the calcium salt method for alkaline phosphatases were obtained. In the gastrodermis intense cytoplasmic and vacuolar activity was seen in the same specimens exhibiting weak esterase and acid phosphatase reactions. At such times the anterior half of the intestine showed a stronger reaction than the posterior, confined mostly to the proximal parts of the columnar cells. Other individuals, and especially those showing some evidence of weak arylamidase activity, possessed alkaline phosphatases throughout their gastrodermal length. The staining intensity was greatest in these animals, appearing equally strong in both food vacuoles and cytoplasm.

As with *Baseodiscus*, prolonged incubation in the Tween medium irregularly resulted in a brownish vacuolar deposit, absent from control sections, appearing in many of the gastrodermal food vacuoles. This reaction may indicate the occurrence of intracellularly acting lipases, apparently effective at the same time as both esterases and arylamidases. No evidence of other sites of enzymatic activity could be found in the gut.

Food reserves. The principal food reserves are fat droplets, filling the intestinal columnar cells in freshly collected animals. They range from minute globules, less than 1 μ in diameter, up to amorphous masses 3–4 μ across. Lesser amounts of fat deposits can also be found in the body wall muscle layers and in the anterior proboscis epithelium. Glycogen, as small particulate deposits, is confined to the gastrodermis, where it is mainly restricted to the distal half of the columnar cells.

Enzymes of other tissues. Enzymic activity was found in several other sites within the three species investigated.

Strong arylamidase activity is present in the blood vascular system, mostly confined to the endothelial layers of the vessels but, on occasion, also showing within the blood fluid. The occurrence of these enzymes in nemertean blood systems seems likely to be universal.

Arylamidases were also found at two other sites. In *O. affinis* a weak reaction was sometimes seen in parts of the body wall musculature, and in *O. erneba* whole mount preparations resulted in a darkly staining band of activity extending across the stylet bulb just posterior to the central proboscis stylet.

Esterolytic activity appeared widespread in several body tissues, occurring strongly in all three species in the proximal epidermal regions, less intensely as a thin distal epidermal band, in the basal parts of many gland cells in the anterior proboscis epithelium (Fig. 9), and in ganglionic cells surrounding the lateral nerve cords. At these sites enzymic activity was consistently demonstrable by both the α-naphthyl acetate and indoxyl acetate methods, and appeared to be independent of the nutritive state. In *O. affinis* incubation in the indoxyl acetate medium gave a faint positive reaction in parts of the body wall musculature; it is possible that this activity can be related to the arylamidases demonstrated at the same site in this species.

In general acid phosphatases at sites other than the gut parallel the distribution of esterases, particularly in the epidermis and proboscis epithelium. The proximal epidermal regions especially give a strong positive reaction to the Burstone naphthol phosphate technique.

Alkaline phosphatases were weakly evident in the proximal epidermis of all three species and, in *O. affinis,* also showed in some regions of the principal musculature. A strong reaction for these enzymes was obtained in the posterior proboscis epithelium, and a weak or negative response was seen forming a thin distal band of activity in the epithelium of the anterior proboscis.

DISCUSSION

Gibson (1972) comments that nemertean worms are, for the most part, carnivorous or scavenging macrophages. The *Ototyphlonemertes* species, although in the present investigation consistently refusing to accept either whole or squashed naturally associated psammobiontic polychaetes, turbellarians or other macroscopic fauna under laboratory conditions, clearly possess scavenging habits since they respond so rapidly to the fish meat baiting method of collection. They do, in fact, show evidence of wide dietary preferences; Corrêa (1948) comments that *Ototyphlonemertes* do not apparently live exclusively by scavenging and cites an example of *O. brevis* with a recently eaten crustacean in its gut. Subsequently, Corrêa (1950) used *O. brevis* for feeding experiments in the laboratory, using as food polychaetes, crustaceans and portions of striated muscle from *Armadillidium vulgare.*

No identifiable food remains were found in any of the *Baseodiscus* specimens studied but, as judged from the structure of the gut and the nature of its associated enzymic complement, this species too will be found to be carnivorous and/or scavenging in its habits.

Previous studies on nemertean digestive physiology (Jennings, 1960, 1962a; Gibson and Jennings, 1969; Jennings and Gibson, 1969; Gibson, 1970) have demonstrated that, although the basic sequence in the digestion of food material is the same for all species, interspecific differences in the nature and location of enzymes can be detected which may be related to systematic position, diet and mode of life. The evidence gleaned from the current histochemical studies shows that in all four species the intestinal gland cells obviously synthesize and secrete enzymes for initial extracellular digestion, the subsequent stages being carried out intracellularly in food vacuoles and involving other enzymes of cytoplasmic origin. Since lysosomes appear to be invariably present in animal cells and are known to contain a whole range of hydrolytic enzymes optimally active under acidic conditions (Duve, 1967), it seems reasonable to suppose that intracellular digestion is at least partly due to lysosomal enzymes. Future ultrastructural studies are needed to provide more detailed information about enzyme localization in nemerteans.

The specific differences seen between the species require some further consideration. The absence of demonstrable carbonic anhydrase activity from the foregut of *Baseodiscus delineatus* is unusual, and has not been reported before for anoplan nemerteans. Similarly, the occurrence of esterase activity in many of the foregut glands in this species has not been found in any other nemertean, and it is tempting to suggest that the two features are in some way related. It is clear that during the ingestive phase both mucus and esterase producing glands in the foregut discharge their contents. In anoplan nemerteans foregut secretions are involved in killing live prey, in establishing the appropriate intralumenar pH for subsequent extracellular proteolysis, and in facilitating swallowing by the lubricative action of mucoid products. Initiation of suitable lumenar conditions for the early stages of intestinal digestion must, in *Baseodiscus*, be achieved by a mechanism that does not involve carbonic anhydrase enzymes, as found in several hoplonemertean species including *Ototyphlonemertes*. The role of the esterase secretions in the foregut may in some way be concerned in this mechanism; a more plausible explanation of the part played by these enzymes, however, is that the animals possess carnivorous feeding habits similar to those found in many triclad turbellarians, and that the enzymes are used in the penetration of the prey's body wall and early disruption of its body contents. Pharyngeal gland cells secreting endopeptidase enzymes for this purpose are found, for example, in *Polycelis* and *Orthodemus* (Jennings, 1959, 1962b). An extracorporeal histolytic phase, essential to the feeding mechanism, has been described for the hoplonemerteans *Amphiporus lactifloreus* (Jennings and Gibson, 1969) and *Nipponnemertes pulcher* (Berg, 1972), although in these species the secretions responsible for the breakdown of prey tissues are believed to be derived from the proboscis rather than the stomach.

The remaining stages of digestion in *Baseodiscus* follow the usual heteronemertean sequence. Gastrodermal gland cells discharge enzymes for the extracellular breakdown of food material, which is then phagocytosed by the columnar cells and completely digested intracellularly in food vacuoles. Early intracellular digestion involves esterases and acid phosphatases, the final stages utilize arylamidases and alkaline phosphatases. In the gastrodermis the esterases observed probably represent endopeptidase activity for, although appropriate activators and inhibitors were not available for use in the study, there is no logical reason to suppose that enzymes of other groups are synthesized by the gland cells. With the exception of the unselective microphage, *Malacobdella*, in which the intestinal gland cells apparently secrete carbohydrases (Gibson and Jennings, 1969), all nemerteans depend primarily upon endopeptic enzymes for extracellular proteolysis, including those hoplonemerteans in which the precise nature of the gland secretions has not been established.

Ototyphlonemertes brevis alone amongst the species investigated from this genus is known to be actively carnivorous (Corrêa, 1948, 1950) but, from a comparison of their complement of digestive enzymes and closely similar gut morphology, there is every reason to suppose that the present species possess at least related feeding habits.

Mucus and carbonic anhydrase were not demonstrated in any part of the gut, and in this respect *Ototyphlonemertes* parallels other marine enoplans (Gibson and Jennings, 1969; Jennings and Gibson, 1969; Gibson, 1970). Only in the freshwater hoplonemertean *Prostoma rubrum* has carbonic anhydrase activity been located in the foregut. In other forms the intense basophilia shown by so many of the stomach glands, as found in all three *Ototyphlonemertes* species, is taken to indicate that a major proportion of the stomach secretions are acidic in nature.

The demonstration of esterase activity in the gastrodermal gland cells has not before been reported for any hoplonemertean species. Corrêa (1950) reported that immediately after food was taken into the intestine the epithelium became thinner, interpreting this phenomenon as a symptom of secretion although it might also be explained as due to stretching of the gut wall for the accommodation of the food. Her assumption was supported by evidence of extracellular digestion and, since the animals are carnivorous, the esterase activity seen in the present species must be indicative of endopeptic proteolysis. This conclusion is supported by Corrêa's experiments involving the rapid liquefaction of crustacean muscle fragments by artificially freed gland cell contents. Further, her studies strongly suggest that the enzymes are normally present in the gland cells in an active state and do not require unmasking by foregut secretions before they can be effective.

The apparent absence from the gastrodermis of endopeptidase-secreting columnar cell vesicles, previously believed to be a typical hoplonemertean character, is not altogther surprising when the gland cells themselves contain enzymes of this group. No nemertean species has yet been shown to possess both sites of endopeptic synthesis in its gastrodermis, and the earlier interpretation that columnar cell inclusions represent food vacuoles undergoing early intracellular digestion seems justified. After the phagocytosis of food material, intracellular digestion appears to follow the usual nemertean sequence and involves the normal complement of enzymes. The small amount of evidence for arylamidase activity, and its absence from *O. affinis,* may be simply due to the fact that animals were not studied at the appropriate digestive phase. These enzymes, in the gastrodermis, cannot at other times be visualized histochemically. If, in fact, *Ototyphlonemertes* usually ingests a large-sized meal (*e.g.,* see Corrêa, 1950, Plate IV, f. 16–17, 19–20) it might be reasonably expected that the duration of digestion is accordingly prolonged.

The patterns of digestion in the four Brazilian species thus show features not found in previous studies, although they fit perfectly into the broad concept of digestion in this phylum. *Baseodiscus* differs from other heteronemerteans in its possession of esterase enzymes in and absence of carbonic anhydrase from the foregut, physiological modifications which may be related to its possible feeding habits. The *Ototyphlonemertes* species, on the other hand, fall intermediate between known patterns of digestion in that their foregut is typically hoplonemertean in form and function, whereas their intestinal physiology is like that found in anoplan nemerteans. These differences indicate that very many more species need to be studied before an overall picture of digestive physiology in the phylum as a whole can be built up.

The food reserves in the four Brazilian species studied appear to show no features not previously found in other free-living forms. Fat deposits form the principal reserve store, with glycogen accumulation seemingly playing only a supportive role.

Of the enzymes found at sites other than the gut arylamidases are consistently present in the blood vascular system. They are here presumably involved in the circulation of peptides about the body, as fully discussed by Gibson and Jennings (1967).

Esterase activity was also found in all species located in the epidermis, ganglionic cells of the nervous system and proboscis epithelium. Weak alkaline phosphatase activity too occurred in the epidermis, with additionally acid phosphatases appearing at this site and in association with proboscis epithelial esterases in the *Ototyphlonemertes* species. Epidermal enzymic activity is likely to be concerned either in the absorption of nutrients from the environment, a mechanism initially proposed by Fisher and Cramer (1967) but subscribed to by Jennings and Gibson (1969), or in the secretion of mucoid and other substances which may play some defensive role against potential predators, as outlined by Gibson (1972). *Baseodiscus* in particular, when handled, discharges copious amounts of a thick and viscid mucus.

Esterases seen in the ganglionic cells of the major nerves and, in *Baseodiscus*, around the brain, are likely to represent cholinesterase activity related to the passage of nerve impulses. Kamemoto (1957) found evidence of extremely high cholinesterase activity in *Prostoma rubrum* compared with that of the marine *Cerebratulus lacteus*, and variations in the localization of these enzymes in different species may depend upon individual physiology. Cholinesterases are also believed to be involved in some aspects of muscle physiology (summarized by Gibson, 1972), and esterases found in the body wall and proboscis musculature are probably concerned with this component of the body metabolism. The significance of the weak arylamidase activity found in the body wall musculature of *O. affinis* may similarly be related to muscular function.

Alkaline phosphatases are known to be present in the nephridial ducts of certain nemerteans (Danielli and Pantin, 1950; Jennings and Gibson, 1969), where they are believed to be involved in some way with the modification of nephridial fluid composition. These enzymes in *Baseodiscus* may be expected to possess a related function.

The various enzymes observed at other sites in the different species are not so easily explained. A relationship between alkaline phosphatase and esterase in the ciliated cerebral canal and cerebral organs of *Baseodiscus* may be suggested. There is some evidence in nemerteans that components of the cerebral organs, as well as other tissues, may be involved in some endocrine function, and the occurrence of neurosecretion is well known in lineid heteronemerteans (Lechenault, 1962, 1963, 1965; Bianchi, 1969a, 1969b). It is thus possible that in *Baseodiscus* the cerebral canal and organ enzymes are concerned in endocrine physiology, perhaps through a chemotactic mechanism. Recent studies on the ultrastructure of the cerebral organs of *Lineus ruber* have shown that they may be chemoreceptive, mechanoreceptive or photoreceptive in function (Ling, 1969a, 1969b, 1970), although it is not known with certainly what precise function the organs have. The finding of enzymes at these sites in *Baseodiscus* may, therefore, point towards the cerebral organs possessing a chemoreceptive rather than any other function. This suggestion can, however, be no more than conjecture at this time.

Esterases found in the proboscis epithelium in the four species are likely to be related to some aspect of proboscis secretion, and also is the alkaline phosphatase activity occurring in the posterior proboscis epithelium of the *Ototyphlonemertes* species.

I wish to express my grateful thanks to the Brazilian National Research Council (Conselho Nacional de Pesquisas) of Rio de Janeiro, the University of Sao Paulo and the Royal Society of London for providing the financial assistance which enabled my wife and me to visit and work in Brazil.

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SUMMARY

1. A comparative histochemical study has been made of the nature and location of enzymes associated with digestion in four species of Brazilian nemerteans.

2. The basic digestive physiology is similar in the four species (Heteronemertea: *Baseodiscus delineatus;* Hoplonemertea: *Ototyphlonemertes affinis, O. erneba, O. lactea*), with extracellular acidic proteolysis being followed by phagocytosis and the completion of digestion intracellularly by proteases and lipases acting synchronously. Intracellular digestion is initially acidic and then alkaline, with acid and alkaline phosphatases being associated with the appropriate phase.

3. The proteolytic enzymes responsible for extracellular digestion are produced within the gastrodermal gland cells in all four species. The occurrence of histo-

chemically demonstrable proteolytic enzymes has not before been found in hoplonemertean gastrodermal gland cells. In *Ototyphlonemertes* the glands are physiologically similar to those of anoplan species and, accordingly, the usual hoplonemertean columnar cell endopeptic vesicles are apparently missing.

4. *Baseodiscus delineatus* does not possess demonstrable carbonic anhydrase activity in its foregut, a feature not previously recorded from anoplan nemerteans. In this respect it resembles certain hoplonemertean species.

5. A proportion of the foregut acidophil glands in *Baseodiscus delineatus* contain esterolytic enzymes. These enzymes have not been found in the foregut of any other nemertean species. It is suggested that their occurrence in *Baseodiscus* may indicate an extracorporeal preingestive stage in digestion.

6. The food reserves consist mainly of fat, located in the gastrodermis in all species, but there are supplementary deposits of glycogen at the same site.

7. The occurrence of enzymic activity at locations other than the gut is recorded.

8. These findings are discussed in relation to previous work on the digestive and other physiological processes of nemertean worms.

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ANATOMY OF THE INNERVATION AND NEUROMUSCULAR JUNCTIONS OF THE RADULAR PROTRACTOR MUSCLE OF THE WHELK, *BUSYCON CANALICULATUM* (L.)

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J. C. Herrick (1906, on page 731) suggested that the radular protractors of the channeled whelk "would make fine material for physiological experiments upon molluscan muscle because of their length and regularity." We are in the midst of such experiments using the radular protractor muscles of *Busycon canaliculatum* (Hill, 1958; Hill, 1962; Hill, 1970; Hill, Marantz, Beattle and Lockhart, 1968; Hill, Greenberg, Irisawa and Nomura, 1970; Sanger, 1973; Sanger and Hill, 1972, 1973a). The innervation of the buccal muscles of *Busycon canaliculatum* was described in some detail by Herrick (1906) whose objective was to explain the *modus operandi* of the buccal apparatus. His paper is a model of clarity, giving the results of four to five weeks of dissecting and experimenting in the summer of 1905. We could not improve on his comments, nor upon his description of the macroscopic anatomy of the musculature, but his description of the innervation was incidental to his study of the functional anatomy of the buccal muscles. The time has now come for a more detailed description of the *innervation* not only of the radular protractor muscle but also of the whole buccal apparatus.

In this report we also present the results of our observations on the fine structure of the nerves and of the neuromuscular junctions in the radular protractor muscles. A preliminary report of this work was presented last summer at the General Meetings of the Marine Biological Laboratory (Sanger and Hill, 1973b).

Materials and Methods

Whelks, *Busycon* (*Busycotypus*) *canaliculatum* (Linné 1758), were obtained from the supply department, Marine Biological Laboratory in Woods Hole, Massachusetts. The animals were dissected under a low powered binocular microscope, sometimes with the aid of methylene blue injection of blood vessels. The wall of the proboscis was cut through ventrally and pinned back. Since both muscles and nerves were pigmented, the innervation could be blocked out by careful dissection without staining. The smaller blood vessels were not apparent except after injection through the main vessel of methylene blue solution (0.5% methylene blue in distilled H_2O diluted in sea water to the desired color contrast; usally 1 : 5).

Whelks were fixed and processed for electron microscopy as previously described (Sanger and Hill, 1972). Thick sections for the light microscope, 1 to 3 microns, were cut with an LKB Ultratome III and stained with paraphenylenediamine (Estable-Puig, Bauer and Blumberg, 1965).

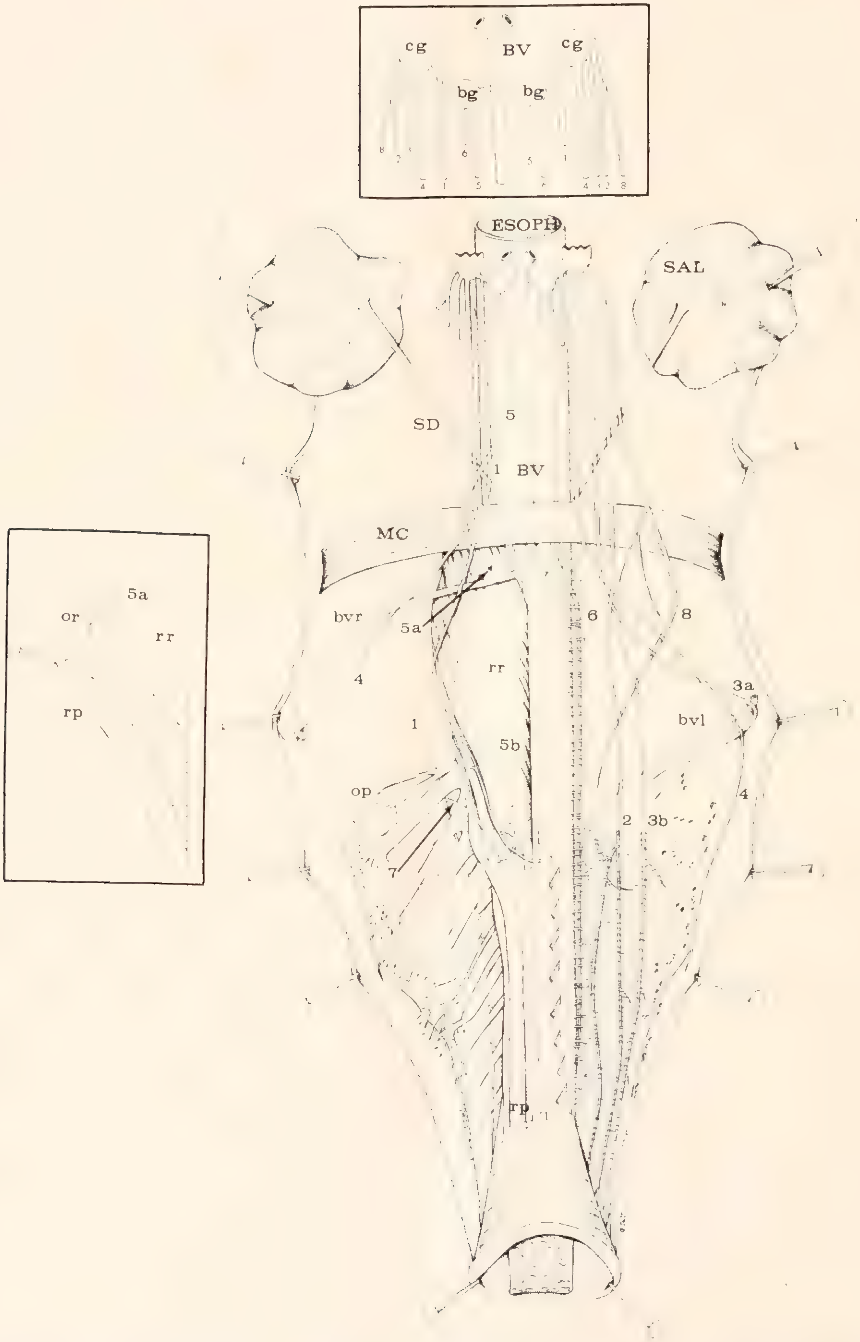
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FIGURE 1. Innervation of the proboscis musculature of *Busycon canaliculatum;* 1–8 = Proboscis nerves. See text for details; BV, chief proboscis artery; ESOPH, esophagus; MC,

RESULTS

We have adhered rigidly to Herrick's terminology (1906) except in the rare instances in which we found that we must disagree with his identification as noted below. [We will *not* reiterate Herrick's description of the macroscopic anatomy of the muscles.]

A semi-diagrammatic representation of the innervation of the buccal apparatus and proboscis wall in *Busycon canaliculatum* based on a dozen or more dissections was prepared and sketched (Fig. 1). We have found individual variations in the finer branches of the nerves, but not in the main trunks. The drawing represents a usual distribution of the finer branches.

The proboscis nerves arise from the buccal ganglia (bg), from the cerebral ganglia (cg) and from the cerebro-buccal connective. In the upper insert of Figure 1 the nerves are numbered for clarity, using Herrick's (1906) *distal* numbers except in the case of nerve 8 (which he did not describe).

Innervation of the proboscis wall is from the cerebral ganglia by nerves 2, 3, 4 and 8. Nerves 2 and 8 arise together from the cerebral ganglia. Nerve 8 soon appears as a branch which first runs ventrally to the muscular cup (MC) and then innervates the white muscle fibers of the proximal dorsal proboscis wall. The other branch, nerve 2, first runs anteriorly (along with all the proboscis nerves) to the muscular cup (MC). All nerves except 6 then pass ventral to the muscular cup (MC). [Most nerves in fact continue more or less on the ventral aspect of the proboscis, but they appear lateral in the drawing, which is a representation of a preparation in which the proboscis wall has been slit ventrally and then pinned out flat, exposing the buccal apparatus]. Nerve 2 then runs anteriorly, ventral to the large median root of the radular retractor (rr). It then runs dorsal to the odontophore protractors (op) along the dorsal proboscis wall, but it is not attached to that wall until it reaches the region of the antrior red fiber layer of the proboscis wall. It then passes under the red fiber layer and gives off branches to the proboscis wall as far as the region of the mouth. Nerve 3 runs along laterally to 2, but somewhat more centrally (ventrally) and provides a branch (3a) which meets and then runs parallel to blood vessel "bvl" or "brr" (for clarity, each nerve is diagrammed only on one side, of the proboscis, but all are symmetrical). All fine branches from 3 supply the proboscis wall. Nerve 4 is the most central (ventral) of the proboscis wall nerves. It is difficult to avoid cutting this nerve when making a ventral incision into the proboscis. To summarize: nerves 2, 3 and 4 arise from the cerebral ganglia; all supply the probscis wall; and all run down to end in the region of the mouth. Since the innervation is symmetrical, and since there is a dorsoventral sequence 2–3–4: the cylindrical tube of the proboscis wall is evidently divided into six longitudinal fields of innervation (probably overlapping).

Innervation of the esophagus is supplied symmetrically from the buccal ganglia by nerve 6 on each side. Nerve 6 runs anteriorly with the other proboscis nerves from a buccal ganglion (bg) to the muscular cup (MC). It then pene-

muscular cup; SAL, salivary gland; SD, salivary duct; bg, buccal ganglion, bvr, right branch from BV; bvl, left branch from BV; cg, cerebral ganglion; op, odontophoral protractor; or, odontophoral retractor; rp, radular protractor; rr, radular retractor.

trates the muscular cup along with the salivary gland duct (SD). After emerging from the muscular cup, nerve 6 runs forward along the esophagus, usually passing under the white muscle fibers that tie the esophagus to the dorsal proboscis wall. On the anterior (red) region of the esophagus, nerve 6 runs with the salivary gland duct (SD) and blood vessel (bvl) providing many branches to the red anterior esophagus.

Nerves 1, 5 and 7 supply the muscles of the buccal apparatus. Since nerve 7 is a branch of nerve 1, this means that the mechanism controlling the movements of the radula is worked by both an outflow from the buccal ganglion (nerve 5) and an outflow from the cerebro-buccal connective (nerve 1) on each side. Nerve 1 arises from the cerebro-buccal connective, usually close to the buccal ganglion, runs anteriorly ventral to the muscular cup (MC), passes ventral to the large median root of the radular retractor (rr), and then runs dorsal to each odontophoral protractor (op). Nerve 7 arises as a large branch of nerve 1, generally just anterior to the most proximal odontophoral protractor. It then branches to innervate the proximal ends of the radular protractors and to supply the transverse muscular sheet which binds the anterior portion of the buccal apparatus. Additionally, a stout branch turns back into the radular retractor (rr). Thus nerves arising with trunk 1 supply odontophoral protractors (op), radular protractors (rp), and a portion (at least) of the radular retractors. Herrick (1906) also reported that nerve 1 supplied the odontophoral pretractors and that nerve 7 (a branch of nerve 1) supplied the radular protractors and the transverse muscle sheet. In his drawing, nerve 7 may also be seen to return in the direction of the radular retractors, although he attributed the innervation of the radular retractors to nerve 5.

Nerve 5 arises from the buccal ganglion, runs with nerve 1 as far as the median root of the radular retractors and then passes within the median root. It soon gives off a branch (5a) which passes laterally in the midst of the median root, giving off small branches, and then enters the odontophoral retractor (or) (see left insert on the diagram). The main nerve (5b) spirals forward in the median root of the radular retractor, giving off small branches and continues anteriorly and dorsally in the radular retractor. Small branches from nerve 5b often seem to anastomose with small branches from the recurring branches of nerve 7, which reenter the radular retractor.

Electrophysiological experiments were carried out (by K. Kuwasawa) to determine if fibers of 5 and 7 actually crossed over. It was found that some motor fibers of 5 did cross over into 7 and vice-versa. The fact that there can be a pathway for excitation at the anastomoses (which are not uncommon throughout the buccal innervation) makes physiological investigation of the innervation more complex, since cutting a given nerve as it emerges from its ganglion would not necessarily isolate it from CNS outflow. Here for example, Herrick has attributed control of the radular retractor to nerve 5, yet stimulation of the innervation of antagonists of the radular retractors can also excite the retractors e.g., stimulation of nerve 7 to the radular protractors).

There is a large artery (BV) entering the proboscis, which gives off two symmetrical lateral branches (bvl and bvr). Injection of methylene blue revealed their continuity with the central arteries in the radular protractors (rp) (Fig. 2)

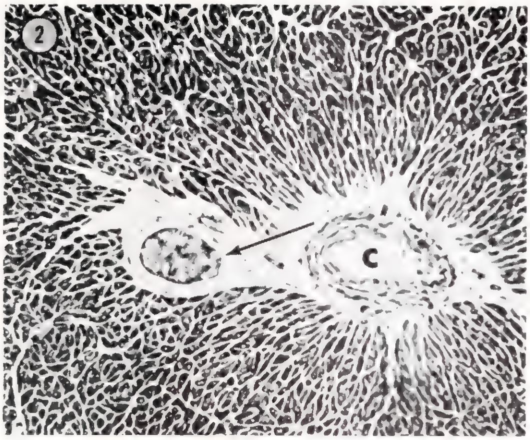

FIGURE 2. A light micrograph demonstrating the relationship of the central blood vessel (c) and main nerve trunk (arrow) in a cross section of the radular protractor muscle.

and in the odontophoral protractors (op). All three vessels remain large until they branch into the tissues around the mouth. All of the buccal muscles are richly supplied with small branches, and since the circulation is open, injection with methylene blue quickly stains the entire space around the muscles in the proboscis.

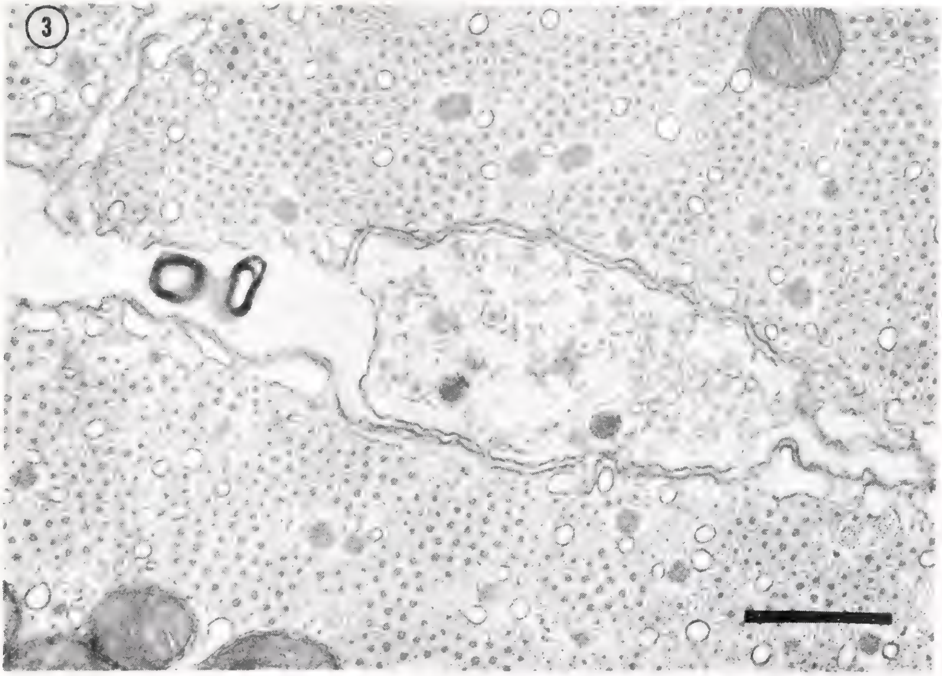

FIGURE 3. A nerve ending between two muscle cells; scale = 0.5 micron.

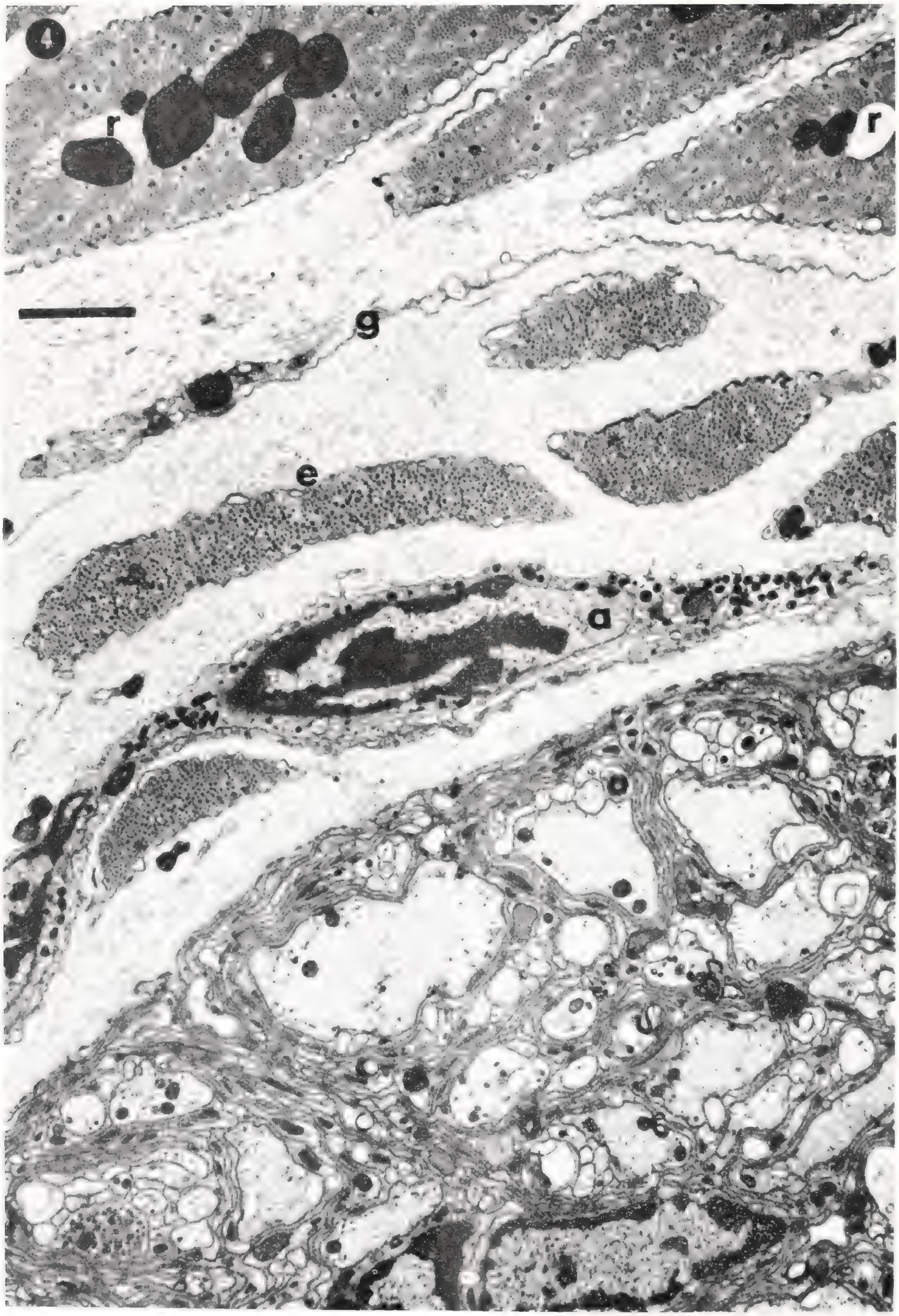

FIGURE 4. An electron micrograph of the nerve trunk surrounded by a discontinous coat of epineural muscle (e), amobocytes (a) and glial cells (g) embedded in collagen. The radular protractor muscle cells (r) are also indicated; scale = 2.0 microns.

Nerves and neuromuscular junctions in the radular protractor muscles: A branch of nerve 7, along with a branch of the blood vessel enter the proximal portion (with regard to the CNS) of the radular protractor muscle which is attached to the odontophore cartilage. The main trunk of the branch of nerve 7 runs parallel to the central artery in the mid part of the muscle (Figure 2). As the nerve proceeds anteriorly towards the attachment of the muscle to the radular sac, it subdivides to form smaller trunks. These smaller trunks continue to subdivide until finally single axons are seen among the muscle cells, where neuromuscular junctions are formed. Occasionally, a single nerve ending is observed forming a junction with two adjacent cells (Fig. 3) (Graziadei 1966).

The main branch of nerve 7 in the radular protractor muscle is surrounded by a discontinuous layer of amoebocytes, glial and epineural muscle cells (Figure 4). These cells are embedded in a thick collagen matrix. The collagen matrix appears to be divided into two unequal layers. The adjacent layer (about 2500 Å thick) is composed of collagenous fibers which for the most part wind circumferentially around the nerve trunk. The outermost layer (about 8 microns in width) is composed of mostly collagenous fibers which are arranged parallel to the long axis of the nerve trunk and epineural muscle cells. The cells embedded in the collagenous neural sheath do not form any continuous barrier to separate the axons from the extracellular space of the radula muscle. The main nerve trunk is about 40 microns in diameter and contains several hundred axons ranging in size from less than a micron to about 8 microns in cross section. The axons contain various arrays of microtubules and neurofilaments. In transverse section, the larger axons are rather irregular in outline and are generally found close to the surface of the trunk. The axons are associated with cells termed sheath cells (Rogers, 1968) or glial cells (Amoroso, Baxter, Chiquoine and Nisbet, 1964; Nicaise, Pavans de Ceccatty and Baleydier, 1968; McKenna and Rosenbluth, 1973). Hereafter in accordance with the nomenclature of Amoroso, Baxter, Chiquoine and Nisbet, 1964, these cells will be referred to as glial cells (Rogers, 1969). In the lamellar extensions of the glial cells are large membrane-bound inclusions which vary greatly in size, density and shape (Fig. 5). The inclusions ranged in diameter from 0.3 to 0.8 microns. These glial bodies are comparable to those described by Barrantes (1970) in the Agentinian slug (*Vaginula soleiformis*). The lamellar extension of the glial cell with its characteristic large membrane-bound vesicles is frequently located near neuromuscular endings. As also noted by Rogers (1969), processes of glial cells not associated with axons are often encountered between the muscle cells (Fig. 4).

Smaller subdivisions of the main nerve trunk are encountered away from the central portion of the muscle bundle (Fig. 5). These smaller trunks (less than 10 microns in diameter) are no longer surrounded by a coat of epineural muscle. The thickness of the collagenous coat and the number of axons in these smaller trunks are also reduced. The maximum diameter of the largest axon is also smaller; generally none larger than one micron. Figure 5 is an illustration of a small nerve branch about 5 microns in diameter which contains about seventy axons.

In large and small nerve bundles granular and agranular vesicles (similar to synaptic vesicles in neuromuscular endings) are observed in various axons (Figs. 5, 6). In these nerve bundles, an axon appears to contain only one type of vesicle. However, both granular and agranular synaptic vesicles are observed in the same

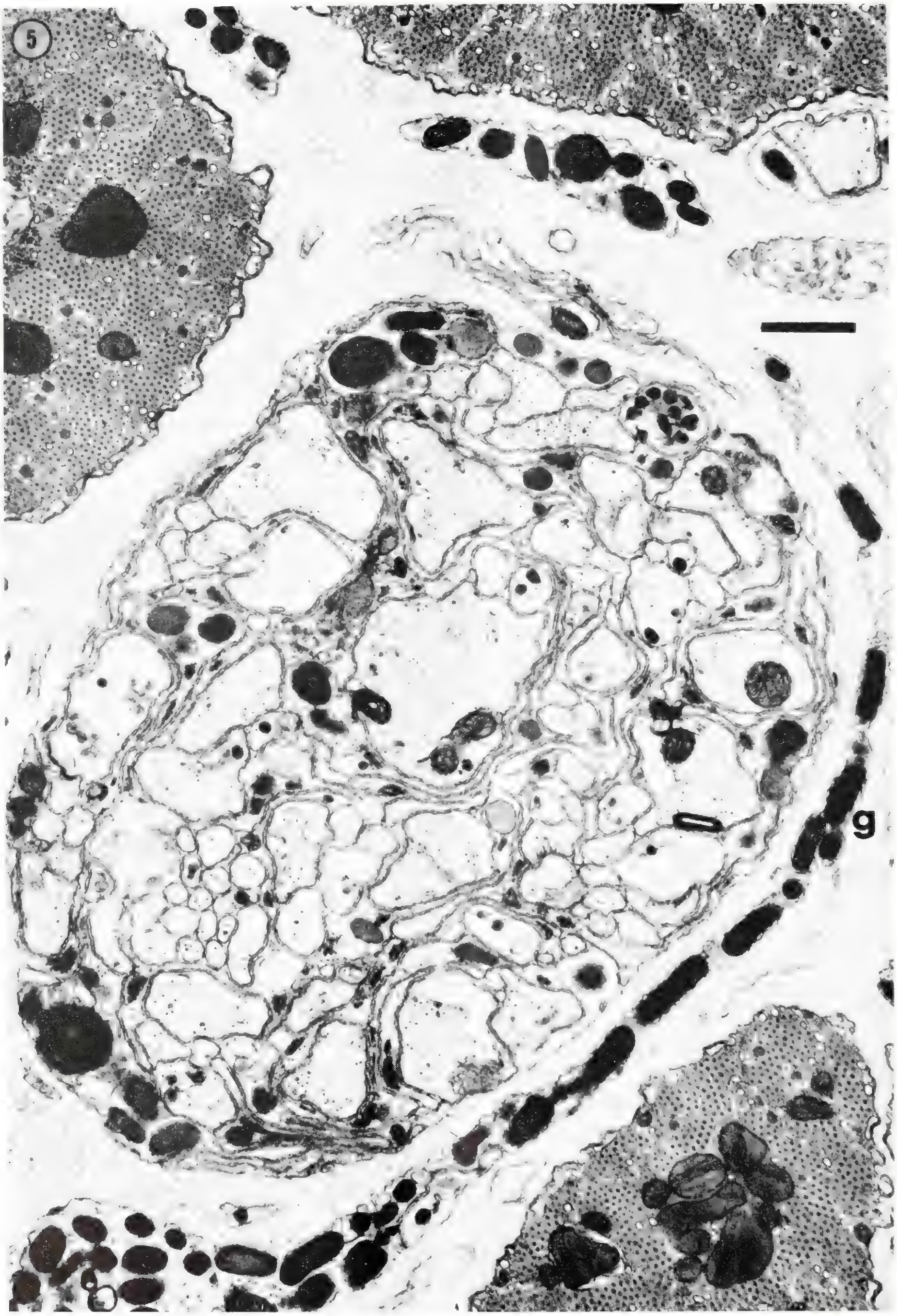

FIGURE 5. A cross section of a smaller nerve trunk among the radular protractor muscle cells. Note the absence of the epineural muscle cells and the meandering process of a glial cell (g); scale = 0.5 micron.

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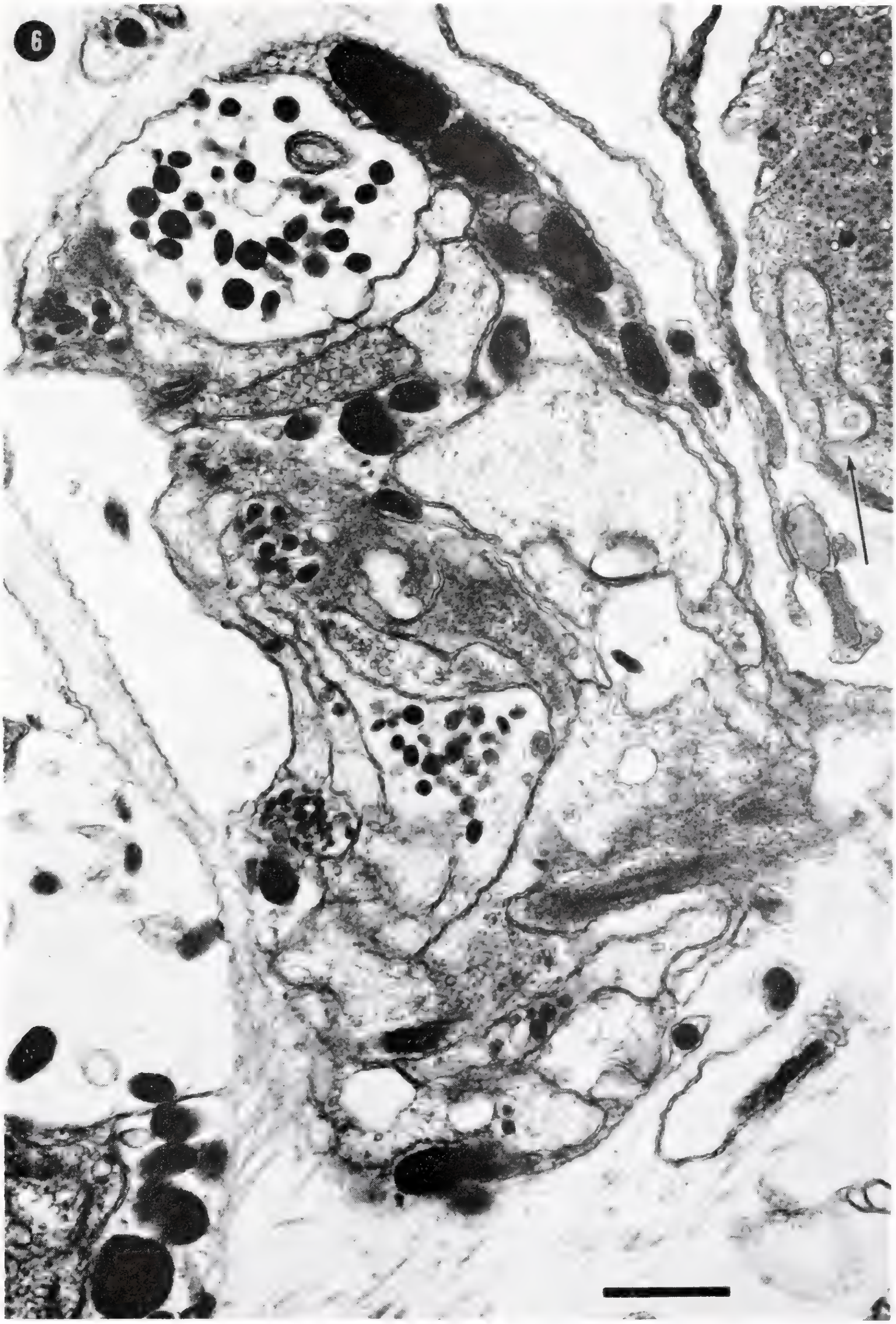

FIGURE 6. Many of the axons in this small nerve trunk contain uniform populations of synaptic vesicles. A nerve ending (arrow) is surrounded by a process of the muscle cell; scale = 1.0 micron.

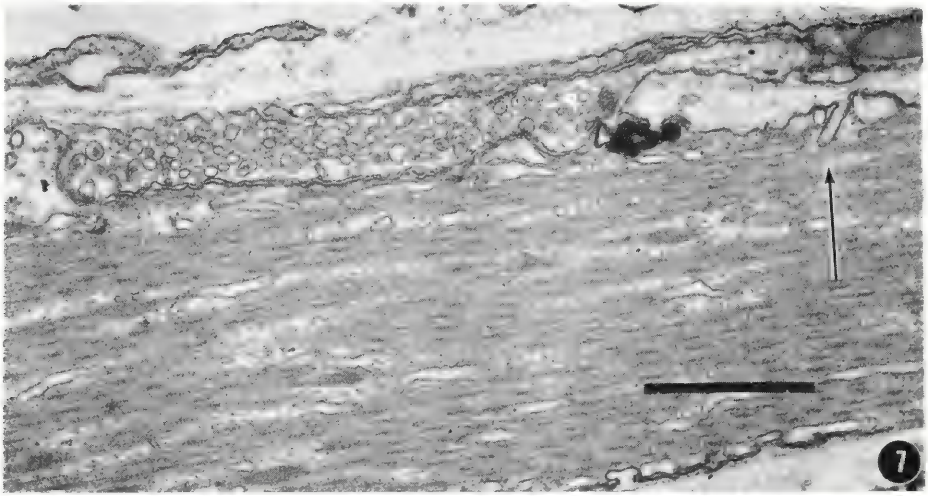

FIGURE 7. The initimate juxtaposition of the nerve ending and muscle cell is illustrated here. A sarcolemmic tubule is also clearly pictured (arrow); scale = 1.0 micron.

neuromuscular junction (Figs. 7, 8, 9). The muscle cell and nerve are separated by a gap of about 150–200 Å. The muscle is intimately associated with the nerve ending but there is no specialized membrane involution in the junctional area such

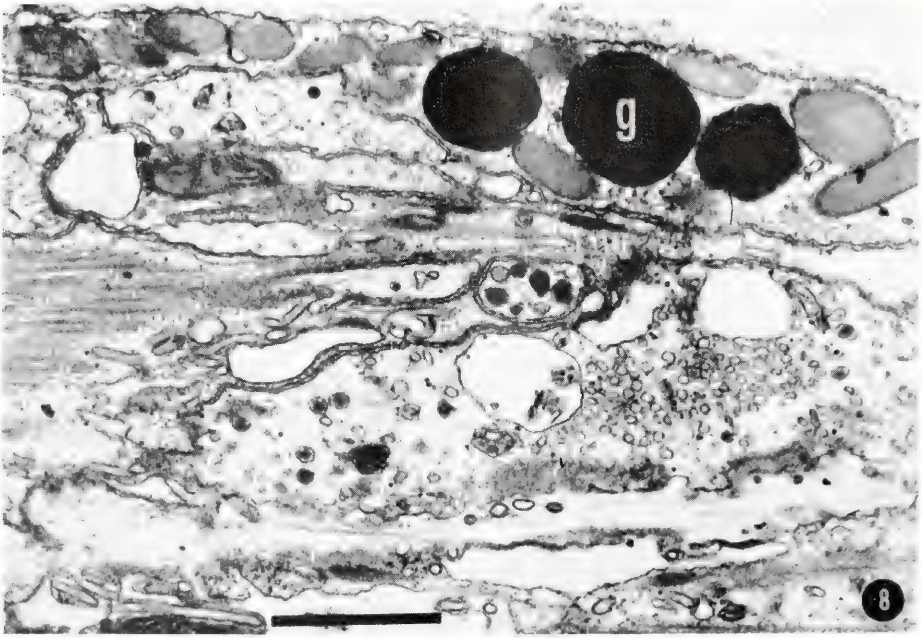

FIGURE 8. A nerve ending containing a mixture of dense and clear synaptic vesicles. The glial cell (g) process contains many large granules; scale = 1.0 micron.

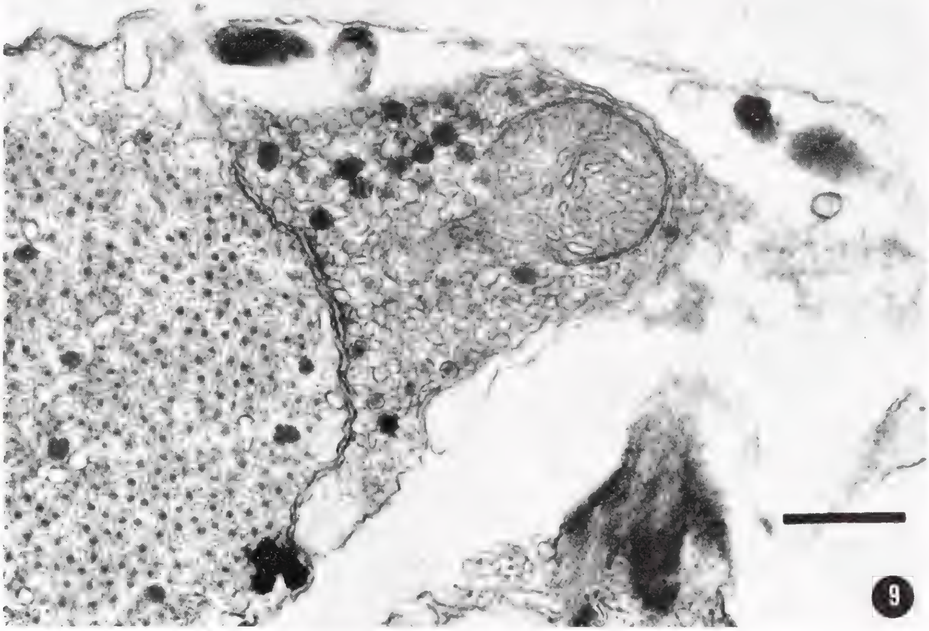

FIGURE 9. A cross section of a nerve ending with a mixture of dense and clear vesicles; scale = 0.5 micron.

as is observed in vertebrate fast twitch skeletal muscle. The association of nerve and muscle is comparable to that observed in vertebrate and molluscan smooth muscle (McKenna and Rosenbluth, 1973). Frequently, part of the nerve ending is ensheathed by a process of the muscle cell it is innervating (Figure 6).

There is a decided segregation in the size of the synaptic vesicles. The agranular vesicles ranged in size from 500 to 1000 Å with a mean diameter of 630 Å (based on the measurement of 400 vesicles). The granular vesicles ranged in size from 800 to 1350 Å with a mean diameter of 970 Å (based on a count of 200 vesicles). The granular vesicles varied in electron density. Some vesicles were completely filled with dense material while others were only partially filled. Nevertheless, both types of granular vesicles had about the same diameter, 800–1350 Å.

The two extremes of neuromuscular junctions are illustrated in Figures 10 and 11. Figure 10 indicates a nerve ending where less than ten per cent of the synaptic vesicles are granular while Figure 11 illustrates an ending where ninety per cent of the synaptic vesicles are granular. Nerve endings with intermediate proportions of granular and agranular vesicles are also observed.

DISCUSSION

A good deal of past attention has been devoted to the function of buccal muscles in the feeding cycle of prosobranch gastropods, and some heat has been generated by past controversies which perhaps did not take sufficient account of the extreme diversity to be found in the anatomy of these muscles in this group. A definitive

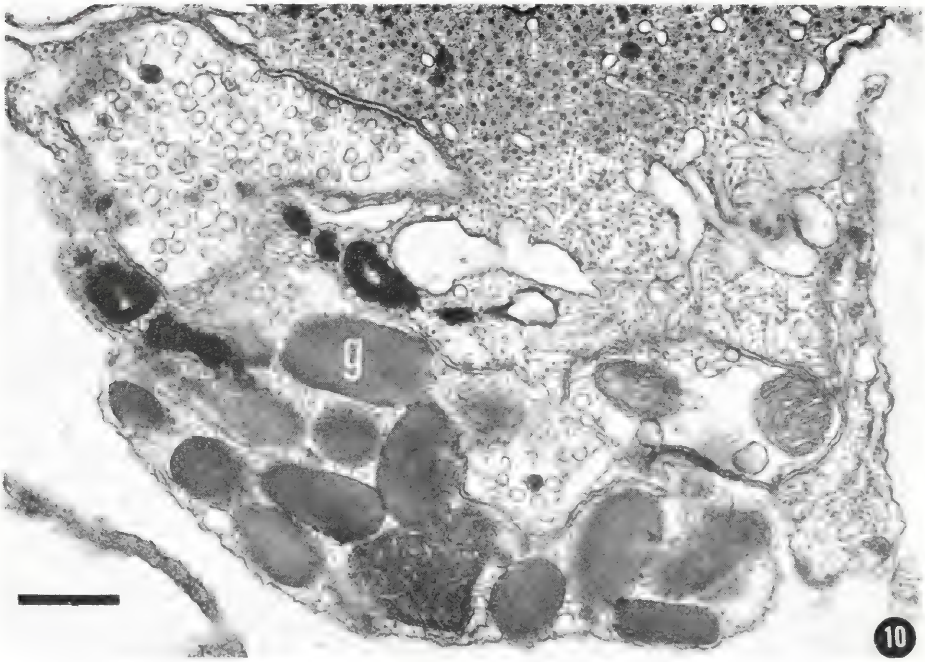

FIGURE 10. A nerve ending where many of the vesicles are clear. Note the intimate association of the glial cell (g) and nerve ending; scale = 0.5 micron.

account of the feeding cycle is available in Chapter 8 of *British Prosobranch Molluscs* (Fretter and Graham, 1962). There are apparent homologies among the muscles in a number of genera, but there are also profound modifications linked with modifications in use of the radula.

The *modus operandi* of the whole odontophoral mechanism of *Busycon* was described by Herrick (1906). As we re-examined the mechanism we felt the need for a redescription of the innervation, which was only one facet of Herrick's work. This was mainly because the responses of the muscles to stimulation of the nerves did not altogether correspond to those to be expected from Herrick's description. We now feel the discrepancies may principally be attributed to the anastomoses described above. Otherwise we have only improved in some details on Herrick's description, but we have provided an integrated picture of the whole buccal innervation.

Hoyle (1964) noted in a review some ten years ago the scarcity of work reported on the ultrastructure of molluscan nerve endings. In the ensuing decade much less than a score of papers have been devoted to this subject (see summary of references in Heyer, Kater and Karlsson, 1973). Even fewer of these studies have been done on systems which are also being investigatd by physiological and pharmacological experiments. The radular protractor muscle of *Busycon canaliculatum* is being investigated in three different ways: physiologically, pharmacologically and ultrastructurally (Hill, 1958; Hill, 1970; Hill, Greenberg, Irisawa and Nomura 1970; Sanger and Hill, 1972; Sanger and Hill, 1973a, 1973b;

Sanger, 1973). Our intention has been to combine the results from these three avenues of experimentation to study regulation of the contractile properties of the smooth muscle at several levels. The radular protractor muscle is of particular interest because it can be induced to show rhythmicity in the presence of acetylcholine and tryptamine (Hill, 1958) and perhaps could be used as a model of cardiac rhythmicity.

The two extreme types of nerve endings observed in this present study appear very similar to serotoninergic nerve endings (containing mostly granular vesicles) and to cholinergic (containing mostly clear vesicles) nerve endings. The nerve endings containing mostly clear vesicles are like those of cholinergic nerve endings in vertebrate smooth muscle (Richardson, 1964). Even there, occasional dense synaptic vesicles were observed by Richardson among the many clear vesicles. These clear or agranular synaptic vesicles might be acetylcholine storage sites while the dense or granular vesicle may be 5-hydroxytryptamine storage sites.

Welsh and Moorhead (1959), using a fluorescence assay method, identified the presence of 5-hydroxytryptamine in the radular muscles of *Busycon canaliculatum*. The concentration was rather low (0.09 μg/g) compared to that in the pooled ganglia (9.2 μg/g), and the origin may well have been from vesicles in the nervous tissue of the radular muscles. This is, of course, speculation since the radular protractor muscle has not yet been studied by subcellular fractionation or by the use of the Falck method. Acetylcholine has not been identified chemically in radular muscles or nerves, although a number of authors have found acetylcholine-equivalent by bioassay in gastropod cardiac muscle (summarized by Welsh, 1956).

The immediate interest of the observation that the nerve endings may have cholinergic and aminergic vesicles lies in the fact that a mixture of opposing synthetic neurohumors, acetylcholine (ACh) and serotonin (5-HT), induce strong maintained rhythmicity in the radular protractor (Hill *et al.*, 1970). Acetylcholine depolarizes and induces a contracture, which is not a "catch" since the muscle relaxes along with the repolarization which follows washing out of the acetylcholine. However, if the muscle is treated with tryptamine while still depolarized with acetylcholine, repolarization is gradual and accompanied by a regular oscillation which induces mechanical rhythmicity. The phenomenon is being investigated, with the objective of discovering the mechanism of the rhythmicity but it may have nothing to do with the natural rhythmic rasping function of the radula, since to all appearances the muscles of the buccal apparatus function in a phasic non-spontaneous motor unit organization (Herrick, 1906). However, it remains possible that acetylcholine may be the excitatory neurotransmitter. When the radular protractor is driven by stimulation of its nerve (Hill, 1962) 10^{-8} M ACh may increase amplitude of twitches by one-third. Michael J. Greenberg, (Florida State University) and his students in the Experimental Invertebrate Zoology Course (MBL) recently found that ACh antagonist blocked twitches caused by stimuli to the nerve (M. J. Greenberg, personal communication). ACh antagonists also block ACh-induced contraction (Hill, 1970). However, tryptamine or 5-HT also increases amplitude of the twitches when the muscle is driven by stimulating its nerve, with a threshold around 10^{-8} M. Furthermore, high concentrations of 5HT (10^{-3} M) predispose the muscle to rhythmicity, in the sense that

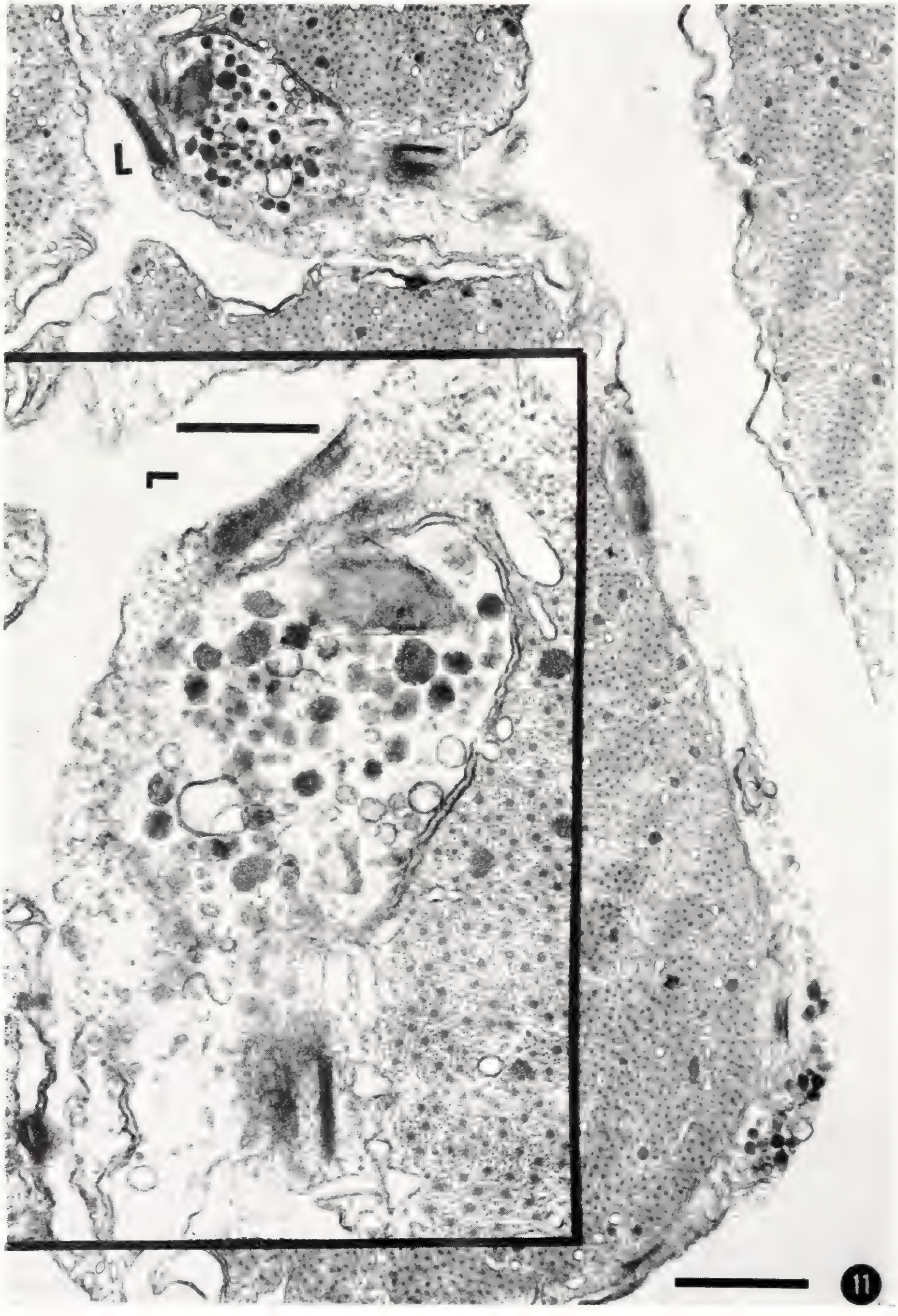

Figure 11. Two nerve endings where most of the vesicles contain dense material. The scale in the large picture equals 1.0 micron. The insert is a higher magnification (scale = 0.5

subsequent stimulation of the nerve or directly of the muscle evokes oscillatory responses, and subsequent stimulation with acetylcholine or KCl may evoke a contracture with superimposed rhythmicity. Thus if the vesicles do indeed contain ACh and 5HT it remains possible that there may be two types of excitatory synapses

The variation in types of synaptic vesicles in nerve endings reported here in the radular protractor muscle, a phasic muscle, is similar to that reported in a "catch" muscle, the anterior byssus retractor muscle (ABRM) (McKenna and Rosenbluth, 1973). Several workers have proposed the existence of two types of nerve endings in that catch muscle. One ending is cholinergic and that nerve ending produces depolarization and contraction, by the release of acetylcholine, which leads to the "catch" state (*i.e.*, the persistent contraction after the muscle has repolarized). A second type of ending is serotoninergic (containing dense vesicles) and by the release of serotonin immediately relaxes the muscle from the "catch" state (Twarog, 1967). In contrast, the radular protractor muscle does not demonstrate any "catch" phenomena—yet it has a similar display of nerve endings as in the "catch" muscle (McKenna and Rosenbluth, 1973). These observations demonstrate a similar ultrastructure of the neuromuscular endings of two dissimilar types of muscles, a "catch" muscle and a phasic muscle.

As indicated by McKenna and Rosenbluth (1973) the mixing of both granular and agranular synaptic vesicles in nerve endings is observed rather frequently in invertebrates. The presence of both types of synaptic vesicles in the same nerve endings has been reported in several other molluscan species (Barrantes, 1970; Dougan and McClean, 1970; Heyer, Kater and Karlsson, 1973). This mixing of different types of vesicles in the same nerve ending raises further questions about their identification and function. Can nerve endings have more than one neurotransmitter? Are the vesicles in the small axons identical to those in the nerve endings? Are some of the clear vesicles in the nerve endings just depleted dense vesicles? Further work-involving serial sections of the nerve endings and small nerve trunks as well as the isolation and identification of the various granules—is certainly needed to elucidate these questions.

We wish to particularly acknowledge collaboration by Mrs. Else Froberg and Dr. Kiyoaki Kuwasawa. The diagram of the innervation is based on dissections by R. B. Hill, D. Spring, L. Vargish, F. Froberg and K. Kuwasawa. The drawing by G. DeVry is based on sketches by E. Froberg, K. Kuwasawa and M. Parmenter.

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micron) of the upper nerve ending. The change in orientation in the insert is indicated by the letter "L."

SUMMARY

1. A detailed and integrated picture of the whole buccal innervation of *Busycon canaliculatum* is presented.

2. The results of our observations on the fine structure of the nerves and of the neuromuscular junctions in the radular protractor are reported and discussed. Each radular protractor muscle is innervated by a nerve arising from the cerebrobuccal connective. The nerve trunk enters the proximal end of the muscle and runs parallel to the long axis of the muscle bundle. A layer of connective tissue and epineural muscle cells surrounds the trunk. Subdivisions of the main nerve trunk branch laterally into the muscle bundle losing their epineural muscle coat. Further subdivisions of the branches produce single axons which can be observed among the muscle cells, but no specialized motor nerve endings were observed. Within the nerve endings are two types of synaptic vesicles: agranular (clear) and granular (dense). The granular vesicles are larger, ranging in diameter from 800 to 1350 Å (mean 970 Å). The clear vesicles vary in diameter from 500 to 1000 Å (mean 630 Å). The ratio of agranular to granular vesicles within a single nerve ending varies widely. When one type of vesicle predominates in an ending, then that ending comes to have a resembance to a cholinergic or to a serotoninergic nerve ending.

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THE DISTRIBUTION OF SIX SPECIES OF GASTROPOD MOLLUSCS IN A CALIFORNIA KELP FOREST

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Kelp forests are common features along temperate shores of much of the world. They are confined to shallow, rocky neritic zones where conditions of light, substrate and surge are suitable. Their productivity is very high, and much organic material flows through complex, but definable trophic structures (Miller, Mann and Scarratt, 1971; Mann, 1973). Spatial structures within these forests also are complex but definable, and many organisms occupy restricted habitats. Drach (1960) distinguished at least 8 types of rock habitats along the coast of Brittany, each with a discrete faunal assemblage. Within the kelp forests of California, vertical rock faces are commonly covered by sponges, anthozoans, byrozoans and ascidians, horizontal rock surfaces at similar depths are covered by algae and motile invertebrates such as decapods, gastropods and asteroids, while the undersides of loose rocks are the habitat for several species of brachyurans, amphineurans and ophiuroids (McLean, 1962, and personal observations). Within the range of the sea otter, rocky crevices are the main habitat of abalones and sea urchins (Lowry and Pearse, 1973).

Algae, in addition to rocks, provide habitats for many organisms. The holdfasts of *Macrocystis* spp. and *Nereocystis luetkeana* support an abundant and diverse fauna consisting primarily of polychaetes, decapods, amphipods, gastropods and ophiuroids (Andrews, 1925, 1945; Ghelardi, 1971). Andrews (1925) found that the stipes of *Nereocystis luetkeana* supported few animals while decapods, amphipods, isopods, gastropods and bryozoans were all abundant in the laminae. Wing and Clendenning (1971) found large numbers of micro-invertebrates associated with *Macrocystis* blades, especially blades that were encrusted with the bryozoan *Membranipora*. Clarke (1971) reported that the mysid *Acanthomysis sculpta* is found almost exclusively in *Macrocystis* canopies and within kelp forests this also appears to be the case for the shrimp *Hippolyte californiensis* (L. Lowry, unpublished data).

In the study reported here, we examined habitat specificity and differentiation among 6 species of closely related gastropods (family Trochidae) on 4 species of kelp forest algae. The snails were chosen because of their apparent ecological similarity (all predominantly subtidal browsing animals of similar sizes) and for ease of collection and identification. The algae were chosen because they provided the snails with a wide range of conditions. The giant kelp, *Macrocystis pyrifera*, is a perennial plant and the dominant alga in the kelp forest studied. Individual plants are anchored to the substrate by a large rhizomatous holdfast from which extend many fronds, each consisting of a central stipe bearing unilateral blades and pneumatocysts. The fronds extend through the entire water column and spread out on the surface to form a thick canopy. The brown alga, *Cystoseira osmundacea*, consists of small (less than 1 meter high), perennial vegetative portions from which massive reproductive fronds rise to the surface of the water from depths of 10 m or more. The reproductive fronds begin growing in mid-winter and break up and disappear by late summer. The vegetative portion of *C. osmundacea* is the main understory plant in our study area (density 1.6 individuals/m^2), while the reproductive portion adds seasonally to the canopy. The other two algae used in this study grow beneath the *C. osmundacea* vegetative fronds and are a major portion of the ground cover algae. These are the brown alga *Dictyoneuropsis reticulata* which has large blades (100 × 25 cm) that usually lie close to the bottom, and the red alga *Gigartina corymbifera* which is similar in growth form to *D. reticulata*, but with somewhat smaller blades (up to 50 cm long).

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Methods and Materials

The study site was located off the northeast side of Point Cabrillo at Hopkins Marine Station, Pacific Grove, California. The area is described in more detail in Lowry and Pearse (1973). Collections were made by divers using scuba equipment during August, 1971, at five locations (depth 6–9 m) in the kelp forest. The plants were chosen arbitarily and all snails were removed from each plant chosen. Snails on *Gigartina* and *Dictyoneuropsis* were simply removed from the algae and put into plastic bottles. Snails from the vegetative and reproductive parts of *Cystoseira* were collected separately. For sampling *Macrocystis*, a line marked in meter intervals was used; snails from each one meter interval up to five meters from the bottom and those from above five meters (including the canopy) were kept separated. Using these procedures, a total of 85 *Macrocystis* fronds (from 6 separate plants), 60 *Cystoseira* plants, 315 *Dictyoneuropsis* blades and 440 *Gigartina* blades were sampled. The snails were then taken to the lab where they were identified, and the maximum basal diameter of each specimen measured to the nearest millimeter.

Results

Sizes and distributions of the species of snails

Calliostoma annulatum (Lightfoot, 1786). The least number of the 6 snail species collected was *C. annulatum*. They also had the smaller modal size (14 mm, see Fig. 1). Twenty-two of the 24 individuals collected were found on *Cystoseira*, 21 of these 22 were on the reproductive portions of the plants. Two individuals were found on *Macrocystis* and none on the ground cover algae (*Dictyoneuropsis* and *Gigartina*) (Table I).

Calliostoma canaliculatum (Lightfoot, 1786). This species was only slightly more common in our collections than *C. annulatum* and it had a very similar distribution. Five individuals were found on *Macrocystis*, but most (21 out of 27) were on the reproductive portions of *Cystoseira* and none were on the ground cover algae (Table I). The size-frequency distribution for this species (Fig. 1) shows

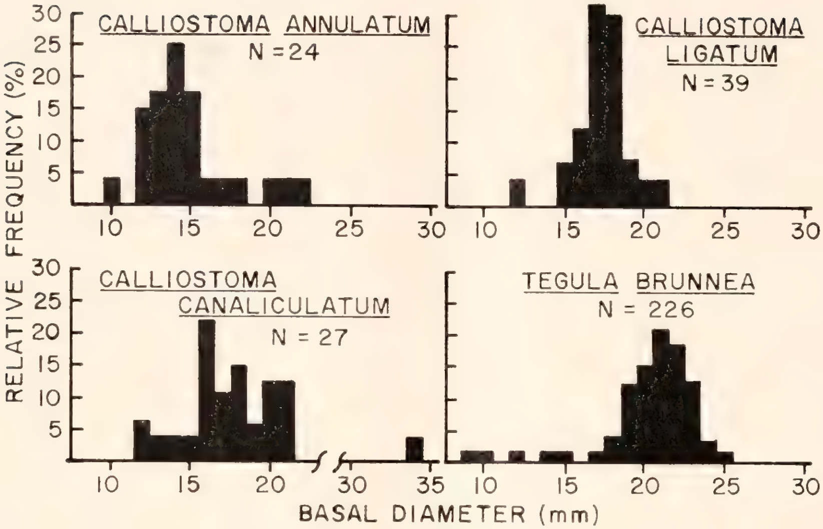

FIGURE 1. Size frequency distributions of the total collections of *Calliostoma* spp. and *Tegula brunnea.* The relative frequency is the per cent of the total collection for a given species that was in each 1 mm size class.

a single mode at 16 mm, and a predominance of large individuals (20–21 mm). The largest snail found (34 mm) belonged to this species.

Calliostoma ligatum (Gould, 1849). Twenty-nine out of 39 individuals of this species collected were found on *Cystoseira,* 13 of these were on the vegetative portions of the algae. Five individuals were found on *Macrocystis* and 5 on *Gigartina* (Table I). The size-frequency distribution (Fig. 1) shows a single mode at 17–18 mm.

Tegula brunnea (Philippi, 1848). One hundred eighty-seven out of 226 *T. brunnea* collected were from *Macrocystis.* Eleven were found on *Cystoseira,* 7 on the vegetative and 4 on the reproductive portions of the algae. Twenty-three specimens were found on *Dictyoneuropsis* and 5 were on *Gigartina* (Table I). The size frequency distribution (Fig. 1) shows a single mode at 21–22 mm.

TABLE I

Per cent of the total collection of each species of snail which was found on a given species of algae. N is the total number of individuals of each snail species collected.

Species	*Cystoseira* vegetative	*Cystoseira* reproductive	*Cystoseira* total	*Macrocystis*	*Dictyoneuropsis*	*Gigartina*	Total	N
C. annulatum	4	88	92	8	0	0	100	24
C. canaliculatum	4	77	81	19	0	0	100	27
C. ligatum	33	41	74	13	0	13	100	39
T. brunnea	3	2	5	83	10	2	100	226
T. montereyi	8	13	21	51	12	16	100	156
T. pulligo	12	19	31	39	21	9	100	404

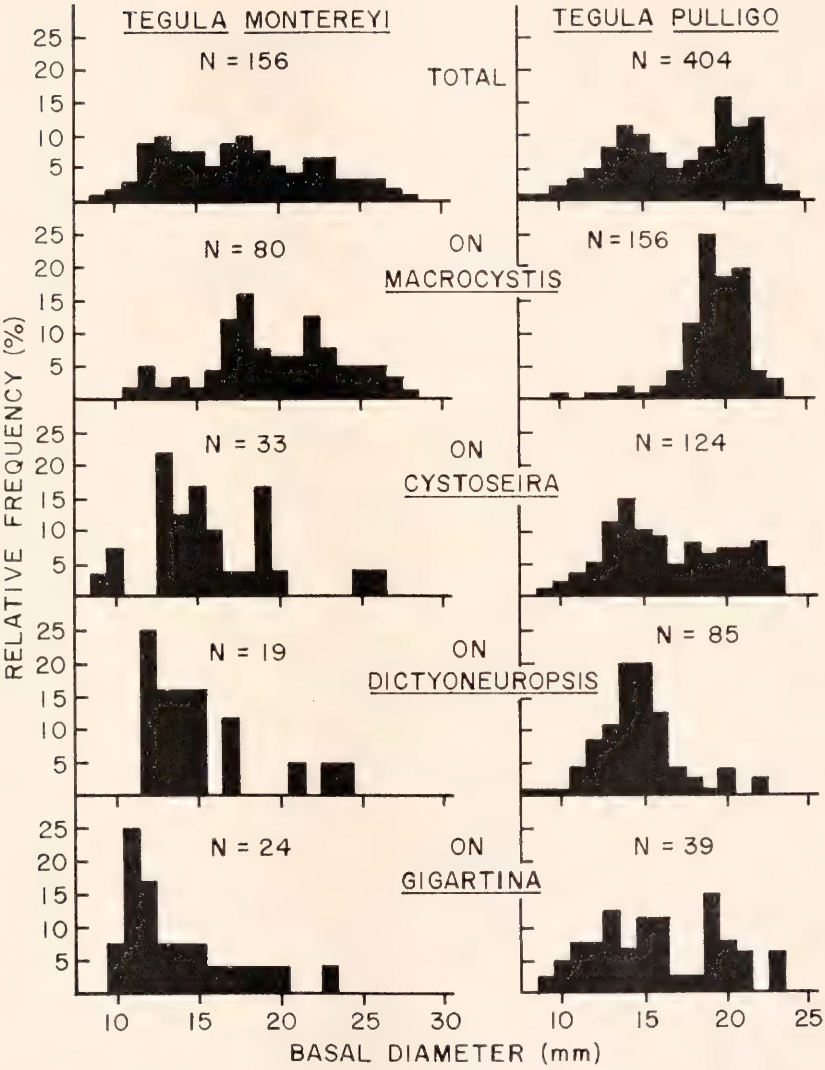

FIGURE 2. Size frequency distributions for the total collections and collections from each species of algae for *Tegula montereyi* and *T. pulligo*. The relative frequency is the per cent of the total collection or the collection from a given algal species that was in each 1 mm size class.

Tegula montereyi (Kiener, 1850). About half (80 out of 156) of the snails of this species collected were found on *Macrocystis*. Thirty-three were found on *Cystoseira*, a few more on the reproductive than on the vegetative portions of the plants. Nineteen were found on *Dictyoneuropsis* and 24 on *Gigartina* (Table I).

The size frequency distribution for the total collection of *T. montereyi* shows 3 modes, one at 13 mm, another at 18 mm and a third at 22–23 mm (Fig. 2). The modes at 18 and 22–23 mm are accounted for by a predominance of individuals of these size classes on *Macrocystis,* while the mode at 13 mm represents smaller individuals which were found mostly on *Cystoseira, Dictyoneuropsis* and *Gigartina.*

Tegula pulligo (Gmelin, 1791). This species was the most frequently collected snail in the study and was found regularly on all algal species investigated. One hundred fifty-six out of 404 individuals collected were on *Macrocystis,* 124 on *Cystoseira,* 85 on *Dictyoneuropsis* and 39 on *Gigartina* (Table I).

The size frequency distribution for the total collection shows 2 distinct modes at 14 and 20 mm, respectively (Fig. 2). As with *T. montereyi,* larger snails were found on *Macrocystis,* while smaller sizes were found on *Cystoseira, Dictyoneuropsis* and *Gigartina.*

Comparison of distributions among algal species

The figures in Table I were used to compute indices of association (Whittaker, 1952) to facilitate comparison of the distributions of the six snail species. The results are shown in Table II. The mean value for the similarity indices calculated for comparisons of species pairs within the genus *Tegula* is 68.3, due mostly to their similar high relative abundance on *Macrocystis.* The mean value for comparisons of species pairs within the genus *Calliostoma* is 66.6, due largely to their similar high relative abundance on *Cystoseira.* These mean values are not significantly different [$t = 0.125$ with 6 degrees of freedom (D.F.)] indicating that although the two genera are found on different algal species, the degree of distributional association found among species of the genus *Tegula* is similar to that found among species of the genus *Calliostoma.* The mean value for comparisons of species pairs which include a species from each of the 2 genera is 32.3. This is significantly lower than comparisons within the genus *Tegula* ($t = 4.126$, 6 D.F.) or within the genus *Calliostoma* ($t = 3.504$, 6 D.F.).

Vertical distribution of Tegula *spp. on* Macrocystis

Figure 3 shows the relative abundance of the 3 species of *Tegula* at various heights on *Macrocystis* plants. At the base of the plants, *T. pulligo* is the most

TABLE II

Indices of association (Whittaker, 1952) calculated from the distribution of snails shown in Table 1. The index for a species pair is calculated as $I_{A,B} = \sum_{i=1}^{5} \min(a,b)$ *where a and b are the values given in Table 1 for species A and B in a given column and i represents the columns used. The vegetative and reproductive portions of* Cystoseira *were considered separately; the values for total* Cystoseira *were not used in the calculations. The index has a maximum value of 100 (exactly equivalent distributions) and a minimum value of 0 (never found on the same species of algae).*

	C. annulatum	*C. canaliculatum*	*C. ligatum*	*T. brunnea*	*T. montereyi*
T. pulligo	31	42	53	56	81
T. montereyi	25	36	47	68	
T. brunnea	13	24	20		
C. ligatum	53	58			
C. canaliculatum	89				

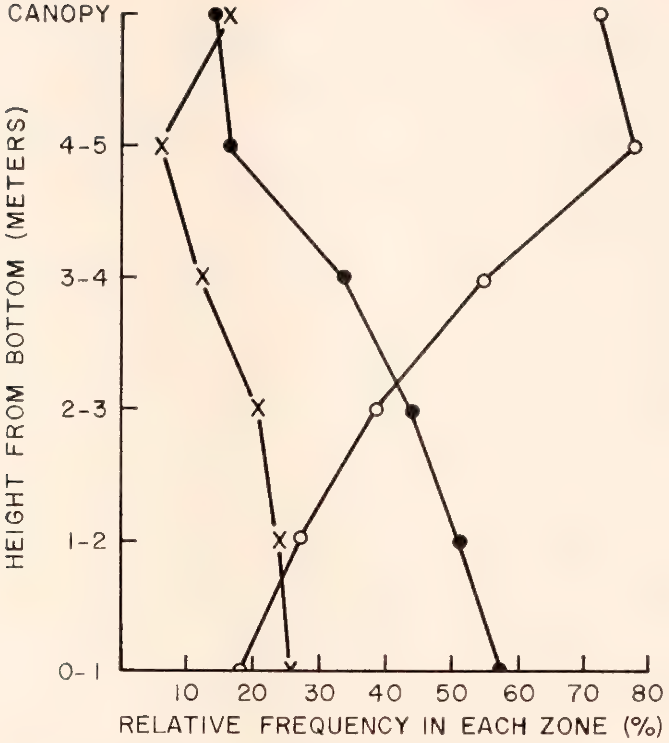

FIGURE 3. Vertical distribution of *Tegula* spp. on *Macrocystis*. The relative frequency is the per cent of the total collection of *Tegula* from a given zone which was made up by each individual species; open circle, *T. brunnea;* ×, *T. montereyi,* filled circle, *T. pulligo.*

common species (56%) and *T. brunnea* is the least common (18%). Moving up the plants, *T. brunnea* increases in relative abundance to become the most common species in the upper portions, comprising over 70% of the population in and just below the canopy. *Tegula pulligo* shows a corresponding decrease in relative abundance to become the least common species in the canopy (14%). *Tegula montereyi* shows little fluctuation in relative abundance, making up between 5 and 25 per cent of the population in all zones.

DISCUSSION

Habitat differentiation among the 6 species of trochid snails we studied has been noted briefly by earlier workers. *Calliostoma annulatum*, among the most beautiful of the West Coast snails, was reported by Keep (1887, page 80) to "crawl up the stems of the sea weed and rest near the surface of the water" during calm weather when specimens could be collected in quantities from boats. Smith and Gordon (1948) reported that all 6 species occur on kelp in the Monterey Bay region. In agreement with our observations, they found *Tegula brunnea* to be "near the surface" and *T. montereyi* and *C. ligatum* "farther down" and "well down the stalks," respectively. They also found *T. pulligo* to be scarcer than the other two species of *Tegula*, which is what we found in the kelp canopy; however, we found *T. pulligo* to be the most abundant trochid snail in the kelp forest and it occurs mainly near the forest floor, which is difficult to observe without scuba equipment.

Observations reported for four different studies in the Monterey Bay region using scuba equipment are summarized in Table III. There is little agreement among these studies with respect to distributions of *Tegula* spp. and *Calliostoma* spp. The kelp forest studied by Minter (1971) is about 2 km southeast of Point Cabrillo in an area of high exposure to surge, scouring and sedimentation. The substrate consists of low shale outcrops which are frequently sanded over. Minter noted the presence of 5 of the 6 snail species used in our study, and only mentioned them as being present on *Macrocystis*. *Cystoseira* and *Dictyoneuropsis* were present at the Del Monte Beach area but not common; *Gigartina* was not recorded. It would appear that either Minter did not examine *Cystoseira* and *Dictyoneuropsis* for snails, or some factor made these algal species less suitable in his study area.

The area studied by Faro (1970) is about 3 km northwest of Point Cabrillo and seems to have substrate and surge characteristics similar to our area. *Macrocystis, Cystoseria,* and *Gigartina* also were present in similar quantities. *Dictyoneuropisis* was not present; however *Dictyoneurum californicum* and *Costaria costata*, two brown algae similar in growth form to *Dictyoneuropsis*, were present. In spite of the apparent physical and vegetative similarities of the two areas,

TABLE III

Reported habitats of Tegula *spp. and* Calliostoma *spp. in Central California*

	McLean, 1962 Carmel Open Coast	Faro, 1970 Point Piños	Minter, 1971 Del Monte Beach	Lowry, McElroy and Pearse, this paper Point Cabrillo
Calliostoma annulatum	Juveniles common on rock walls, maximum development on *Macrocystis*	Common, found on bottom, but more often attached to large brown kelps	Scarce, on *Macrocystis*	Common on *Cystoseira*, especially reproductive fronds, few on *Macrocystis*
Calliostoma canaliculatum	Juveniles common on bottom rocks, maximum development on *Macrocystis*		Fairly common, on *Macrocystis*	Common on *Cystoseira*, especially reproductive fronds, few on *Macrocystis*
Calliostoma ligatum	Abundant on rock walls	Most common *Calliostoma*, distribution similar to *C. annulatum* but less common in turbulent areas	Failry common, on *Macrocystis*	Common on *Cystoseira* vegetative and reproductive fronds, few on *Macrocystis* and *Gigartina*
Tegula brunnea	Juveniles on rocks, adults on *Laminaria* and *Pterygophora*	Most common snail on bottom and holdfasts, seldom found on stipes or blades of kelp	Common on kelp fronds	Abundant on *Macrocystis*, especially in canopy, few on *Cystoseira*, *Gigartina*, and *Dictyoneuropsis*.
Tegula montereyi	Common on *Macrocystis*, *Laminaria* and *Pterygophora*	Common, found attached to kelps, most abundant on stipes and canopy of *Macrocystis*	Common on kelp fronds	Common on all species, larger on *Macrocystis*.
Tegula pulligo	Juveniles on rocks, adults on *Laminaria* and *Pterygophora*			Abundant on all species except *Gigartina*, larger on *Macrocystis*.

the reported distributions of snails are quite different, especially in the case of *T. brunnea* about which Faro (page 66) states that "unlike *Calliostoma* or *T. montereyi,* it was seldom found on the stipes or blades of kelp."

The area studied by McLean (1962) lies about 20 km south of Point Piños, and at the time studied, was a mixed *Macrocystis-Nerocystis* kelp forest. The area appears quite similar to Point Piños with the exception that the dominant canopy-forming alga was *Nereocystis luetkeana,* an anuual plant. The distributions reported by McLean agree somewhat with those given by Faro (1970) but little with the data from our study.

We found most of the individuals of all three species of *Calliostoma* on the reproductive fronds of *Cystoseira osmundacea.* These portions of the plants are seasonal and occur as part of the canopy from the late spring through the summer. In a study at Point Cabrillo carried out in the Fall of 1973, the reproductive fronds of *Cystoseira* were nearly absent, *Calliostoma annulatum* and *C. canaliculatum* were found almost exclusively in the upper portions of *Macrocystis,* while *C. ligatum* was found mostly on ground cover algae (especially *Gigartina*) and the rock bottom (University of California, Santa Cruz, unpublished reports for Neritic Ecology class, 1973). These seasonal differences, at least for *Cystoseira* and *Calliostoma,* may at least partly account for the differences seen among the different studies summarized in Table III. The differences may be further explained by differential responses of the snail populations to the physical, chemical and biological characteristics of the various areas.

The distribution of the snails on the different species of algae may reflect different food preferences of the snails. However, the food of these species of trochid snails has not been described. The species of *Calliostoma* we studied may feed more on sessile animals growing on plants, than on the plants themselves (James T. Carlton, personal communication), and their distributions therefore may be related to the distributions of the animal prey.

The three subtidal species of *Tegula* we studied probably are mainly herbivorous. *T. funebralis* in the adjacent intertidal is known to be mainly herbivorous, and it produces a wide array of enzymes to digest algal products (Galli and Giese, 1959). Moreover, all three species have been fed measurable amounts of algae, particularly *Macrocystis,* in the laboratory (University of California, Santa Cruz, unpublished reports of Neritic Ecology class, 1973). Many other kelp forest herbivores show definite food preferences for some species of algae, particularly for *Macrocystis* (Leighton, 1966). The tendency for the three species of *Tegula* we studied to be on *Macrocystis* suggests that this alga is also a preferred food item for these snails.

The results of between and within genus indices of association comparisons show that the three species of *Calliostoma* are very similarly distributed, being found almost exclusively on *Cystoseira.* The three species of *Tegula* were found primarily on *Macrocystis,* but some individuals were found on all species of algae investigated, and their indices of association are more widely distributed. Character displacement (Hutchinson, 1959) in terms of habitat selection therefore seems effective between the two genera, but less developed within each genus. Interspecific competition, if it exists, consequently might be expected to be most intensive between congeners. However, as indicated by Figure 3, species of the genus *Tegula* are differentiated in their vertical distribution on *Macrocystis* fronds. A similar means of differentiation may exist within the genus *Calliostoma* as *C. ligatum* was common on the vegetative (lower) portions of *Cystoseira* and was found on *Gigartina,* while *C. annulatum* and *C. canaliculatum* were found almost exclusively on the reproductive (upper) portions of *Cystoseira* and were never encountered on the ground cover algae (Table I). Further investigations are needed to elucidate the mechanisms allowing coexistence of these closely related gastropod species.

The bi- and trimodal size distributions of *Tegula pulligo* and *T. montereyi* (Fig. 2) suggest age classes in these snails. It is unlikely, however, that these are annual age classes and that the snails reach the size of 20 mm diameter in two to three years. Paine (1969) found that *Tegula funebralis* in the intertidal grows only about 1–4 mm per year, and 20 mm snails are about 15 years old. There seems little reason to believe that the subtidal species of *Tegula* grow at much higher rates. Rather, the bi- and trimodal size distributions we found probably reflect occasional years especially favored for recruitment, spaced several years apart. Such fluctuations in annual recruitment, with favorable years separated by several unfavorable years are known to occur in other marine animals (*e.g.,* asteroids, Loosanoff, 1964; and echinoids, Ebert, 1968).

McLean (1962) noted that juveniles of several species of snails were found mostly on bottom rocks. Bakus (1974), looking at snails in southern California kelp forests, noted that smaller individuals of *Norrisia norrisi* and *Tegula aureotincta* were located mainly on or in *Macrocystis* holdfasts or on the sea floor, while there were numerous larger individuals of both species on the stipes, pneumatocysts and blades of *Macrocystis.* Although rocks were not looked at in our study, the algae which lie close to the bottom had smaller individuals of *Tegula montereyi* and *T. pulligo* than did the larger, upright species of algae (Fig. 2).

North (1954) observed different size distributions in several populations of the intertidal gastropods *Littorina planaxis* and *L. scutulata.* He explained these size differences as being in part due to a greater ability of small snails to grip the rock substrate, resulting in a greater proportion of small individuals in areas exposed to heavy surge. Frank (1965) found that the intertidal limpet *Acmaea digitalis* shows a behavioral response to environmental conditions which involves an upward movement in the fall and winter followed by a lesser downward movement in the spring. This results in older, larger individuals being located higher in the intertidal. Paine (1969) found that larvae of the snail *Tegula funebralis* settle high in the intertidal, where its primary predator, the sea star *Pisaster ochraceus,* does not occur. When the snails are 5–6 years old, they migrate to a position lower in the intertidal where, as a consequence of the predatory activity of *Piaster* which reduces the density of *Tegula* populations, conditions are more suitable for growth and reproduction. More information is needed before it can be decided whether one of the above mechanisms, or another such as differential growth rates on different algal species or changes in habitat or food preference with age, is responsible for the size distributions observed in our study.

The question of how the distributions reported in this paper are established and maintained also remains. Bakus (1974) noted that a one-day period of heavy surge dislodged all *Norrisia norrisi* and *Tegula aureotincta* from the upper portions of *Macrocystis* plants. The same plants had "abundant" snails one month later.

In his area, Bakus considers the sheephead (*Pimelometopon pulchrum*) the principal predator on snails, particularly smaller individuals on or near the sea floor. Sheephead are not common in our study area; however, several species of predaceous asteroids (*Orthasterias koehleri, Pisaster brevispinus, P. giganteus,* and *Pycnopodia helianthoides*) are common (total density about 0.3 individuals/m^2). Feder (1963) has shown that escape responses to sea stars can influence the distribution of snails in the intertidal. It seems possible that adaptive responses to predators is at least one of the factors responsible for the snail size and species distributions we observed in the kelp forest at Point Cabrillo.

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SUMMARY

1. The summer distribution of 6 species of gastropod molluscs found in a kelp forest is analyzed. From 4 species of common macroalgae, 876 snails were collected.

2. *Tegula brunnea* was found primarily on *Macrocystis pyrifera. Tegula montereyi* and *T. pulligo* were fairly evenly distributed among the 4 algal species. *Calliostoma annulatum, C. canaliculatum,* and *C. ligatum* were all found primarily on the reproductive fronds of *Cystoseira osmundacea.* Indices of association are given which show that the mean distributional associations for pairs of species within the same genus are not significantly different for the 2 genera. When combinations involving members of the two different genera are compared to within genus combinations, the similarity was found to be significantly higher for the within genus combinations.

3. An analysis of populations of *Tegula* spp. on *Macrocystis* showed differences in species compositions at different heights on the plants. *Tegula pulligo* was relatively most common at the bottoms of the plants and least common in the canopy. *Tegula brunnea* showed the reverse trend.

4. A comparison of the size frequency distributions for *T. montereyi* and *T. pulligo* collected from the different species of algae showed that small individuals tend to be on the ground cover algae (*Gigartina corymbifera* and *Dictyoneuropsis reticulata*) and larger individuals on the large, upright *Macrocystis.*

5. Little agreement was found when comparing the distributions found in our study with those previously reported. This lack of agreement is probably due to differences among different areas as well as among different seasons.

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THE CELLULAR ORIGIN OF BIOLUMINESCENCE IN THE COLONIAL HYDROID *OBELIA*

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The ability to produce light is a phenomenon present in many marine organisms. Within the hydrozoan coelenterates the mechanisms that control the production of light are gradually coming to be known and understood. Using the colonial hydroid *Obelia*, Morin and Hastings (1971a, 1971b) presented biochemical information on light emission from a calcium activated photoprotein and a secondary emission from an associated green fluorescent protein via an energy transfer. Physiological mechanisms controlling the multiple flash response in *Obelia* have been examined by Morin and Cooke (1971b, 1971c). The responses were monitored by recording the electrical potentials of an excitation system and by measuring the coupled luminescence of small spots within the animal.

While these previous papers have examined the mechanisms of bioluminescence, little is known of the structure of the photogenic tissues in hydroids. In the present paper we describe some of the structural aspects of the luminescent effector cells in *Obelia*. For the work described in this paper, image intensification and fluoresence techniques were used to show that luminescent and unique green fluorescent sites are identical; and to determine the size, shape, distribution and localization of these luminescent sites. Preliminary reports of this work have been published (Morin, Reynolds and Hastings, 1968; Morin and Reynolds, 1969, 1970).

Panceri's papers (1876, 1877) are the most recent accounts of the sources of bioluminescence in the hydroid form of the hydrozoa. He concluded that luminescence was located as discrete spots in the ectodermal tissues everywhere within *Campanularia flexuosa*. Davenport and Nicol (1955) investigated the sources of luminescence in several hydrozoan medusae and showed that the photogenic material was intracellular, with masses of several thousand cells lying just under the gastrodermis of the marginal canal.

Image intensification has been used to locate the exact sources of luminescence in the firefly (Hanson, Miller and Reynolds, 1969), *Renilla* (Buck, Hanson and Reynolds, 1967), *Noctiluca* (Eckert and Reynolds, 1967), *Pyrocystis* (Swift and Reynolds, 1968), *Gonyaulax* (Reynolds, Hastings, Sato and Sweeney, 1966), and *Obelia* (Morin, Reynolds and Hastings, 1968). Fluoresence techniques have been used for the inspection of luminescent regions in *Mnemiopsis* (Harvey, 1925; Harvey and Marfey, 1958), certain annelids (Nicol, 1953, 1954), *Noctiluca* (Eckert and Reynolds, 1967), *Aequorea* and other hydromedusae (Davenport and Nicol, 1955), and in the pennatulids: *Pennatula, Pteroides, Veretillum* (Titschack, 1964, 1966), *Ptilosarcus, Renilla, Stylatula, Acanthoptilum* and *Virgularia* (Morin and Reynolds, 1970; Morin *et al.*, in preparation).

MATERIALS AND METHODS

Obelia geniculata (L.) was obtained and cultured according to the method of Morin and Cooke (1971a). The luminescent sites were examined using three methods: (1) autophotography by means of image intensification, (2) fluorescence microscopy and (3) histology which consisted of fixation by means of freeze-drying with subsequent embedding and sectioning for examination with the fluorescence microscope.

Autophotography

Flashes of light for autophotography were evoked by applying 0.5 to 5 msec duration square pulses from a stimulator through a pair of fine, closely spaced silver wires placed across an upright of *Obelia* (Morin and Cooke, 1971b).

The physical arrangement of the image intensifier consisted of a microscope (American Optical) supplemented with a beam director (Zeiss) such that the field could be directed to the oculars for a direct view, to a camera for direct photography, or to the photocathode of the image intensifier tube for intensification and (a) photography at a second camera or (b) recorded on magnetic tape with simultaneous television monitoring. Technical details are given elsewhere (Reynolds, 1972). Magnifications of 3.5 ×, 10 × and 55 × which gave fields of 4 mm, 1.5 mm and 0.25 mm diameter respectively, were used.

Rear illumination photographs were taken by light transmitted from below the microscope stage which was directed to the image tube cathode and recorded.

Image intensification provided a direct means of photographing very low light levels. The luminescence was too weak to be recorded on film without such image intensification. The image intensifier tube gain was varied from approximately 10^4 to 10^6, depending on the magnification and light output of the specimen. Even with highest numerical aperture objectives used, only the order of a few per cent of the light was collected and transmitted through the microscope, so that in general, high image tube gains were required.

Sources of image noise which produced small spots on the developed film were (1) thermal electrons from the cathode, (2) background grains in the film (fog) and (3) scatter and reflection within the specimens, chamber and optical system. The large difference in size between the luminescent spots and the small noise spots reduced this problem to a negligible level (Fig. 1).

Fluorescence microscopy

Fluoresence was photographed through the image tube by means of rear illumination combined with the proper filters. A blue (460 nm) interference excitation filter was placed between the light source and the specimen, and a green (507 nm) interference pass filter was placed between the specimen (after the optics) and the image tube. The resulting image with its fluorescent spots was photographed on the camera behind the image intensifier for comparison with the pictures of the luminescence taken of the same field.

Still photographs of the image tube anode were taken using an f/1.9 lens and Polaroid film (ASA 10,000). With the room totally darkened, the shutter was opened, the specimen was stimulated and then the shutter was mechanically closed by the observer of the image tube anode, after the flash had been seen (usually 1–2 seconds).

A Leitz ultraviolet microscope with 2.5 ×, 10 ×, 54 × (oil immersion) and 94 × (oil immersion) objectives was used for fluorescence microscopy without the image intensifier. Ultraviolet illumination from a 200 watt, high pressure mercury lamp was used with a dark field condenser in order to maximize the observed fluoresence. Exciting light passed through a heat absorbing filter (Leitz BG 38) and an ultraviolet pass filter (Leitz BG 12) with a peak transmittance at approximately 400 nm. A barrier filter (Leitz K510) removed wavelengths below 500 nm. Kodachrome Tri-X black and white film was used for photography.

Freeze-drying methods

The freeze-drying method was similar to that of Rude (1966). The specimens were pinned to a planchet in sea water, dipped into distilled water for about one second in order to remove external salts, drained briefly on filter paper, and then plunged into isopentane cooled by liquid nitrogen (−160° C). The frozen colonies were transferred to a freeze-dry appartus and dried for about three days at a pressure of 2 μ Hg and an outside temperature of −40° C. The specimens were then slowly brought to room temperature. The specimens were placed in Maraglas embedding medium in a vacuum desiccator for several hours; they were then transferred to a 40° C oven for about seven hours until the Maraglas hardened. Seven μ serial cross sections were made on a Spencer A.O. microtome with metal knives. The serial sections were mounted in Entellan, a non-fluorescing mounting medium, on microscope slides and examined with the fluorescence microscope.

RESULTS

Visual appearance of the luminescence

Stimulation of *Obelia geniculata* evoked light which emanated from small points within the colonies. Within a given microscope field the light showed distinct multiple flashes in response to individual stimuli. It was difficult to determine visually the precise source of the luminescence because of the flickering and relatively weak light. It was not possible by visual means to be certain if individual spots were flickering or if the flicker was a consequence of sequential luminescence of different spots, possibly along the length of the colony.

Evidence, presented from photometric responses of single luminescent sites (shown by fluorescence), indicated that single spots did flash repetitively (Morin and Cooke, 1971b, 1971c). Autophotography and fluorescence microscopy provided further evidence for repetitive flashing of single spots and information about the general location of the luminescent sites.

Autophotography and fluorescence of the luminescent sites

General characteristics of the luminescent sites. The usual pattern of luminescence in *Obelia geniculata,* demonstrated by single frame autophotography, is shown in Figure 1. Figure 1A-C shows a 3.5 × field of an upright with (A) rear illumination, (B) luminescence and (C) both almost superimposed. An enlarged field (10 ×) of the central portion of figure 1A-C is shown in Figure 1D-F with the same format. The photographs show that the luminescent sites are discrete spots of variable size. The spots are located in the uprights and pedicels and not in the hydranths; the spots remain fixed in their spatial patterns, at least between successive photographs spaced several minutes apart. In addition to this constant pattern of organization, other photographs showed that the stolons contain luminescent spots and that medusae within the gonangia show no luminescence upon stimulation of the colony, although mature medusae are capable of emitting light.

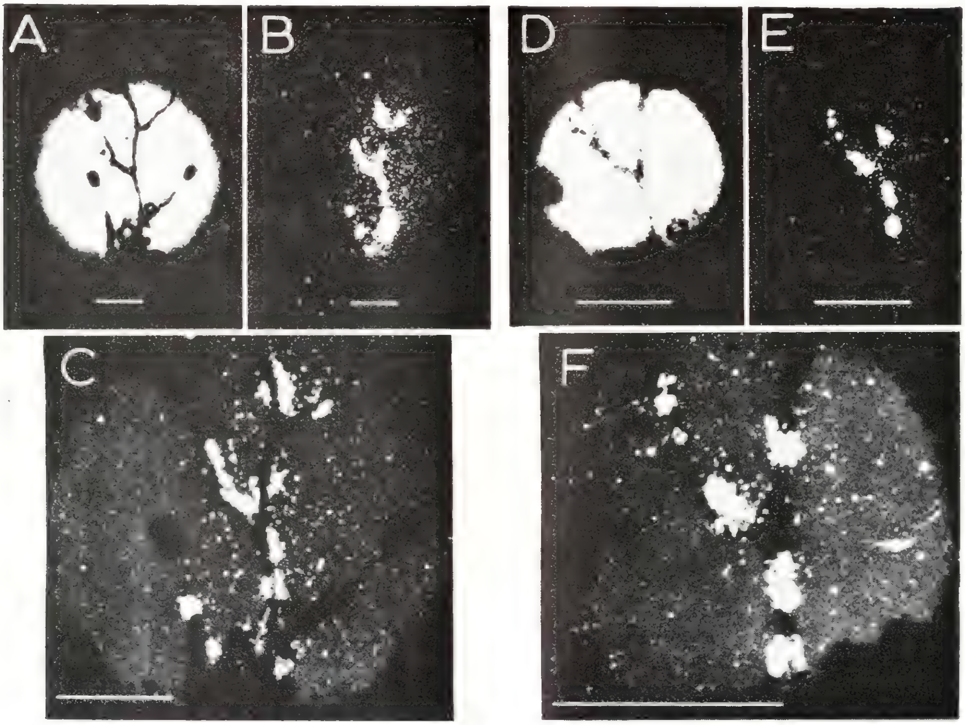

FIGURE 1. Image intensifier photographs of *Obelia geniculata* bioluminescence: (A–C). Low power (3.5 ×) photographs showing (A) rear illumination of upright, (B) autophotograph of upright luminescence and (C) superposition of rear illumination (A) and autophotograph (B). (D–F), same colony but at a higher magnification (10 ×) taken a few minutes after (A–C); same format. Bars indicate 1 mm. Scattered small spots in (B) and (E) are image noise (see text for details).

Correspondence of fluoresence with luminescence. Biochemical evidence indicated that the luminescent sites could be located and observed using a method which exploits the fluorescence charcteristics of the emission system when excited with blue light (Hasting and Morin, 1969a, 1969b; Morin and Hastings, 1971b). Such a method involved excitation of the luminescent sites with 460 nm light and examination of the emitted light using a barrier filter to exclude wavelengths shorter than 500 nm. The sites revealed by this method fluoresced a bright green color ($\lambda_{max} = 508$ nm). Yellow-green fluorescence from small particles (a few microns) was also observed within the colonies, especially in well nourished ones. There was no correspondence between these latter fluorescent sites and the luminescent sites. Epiphytic diatoms attached to the perisarc of the colonies displayed the characteristic red color of chlorophyll fluorescence.

The direct correspondence between luminescence and fluorescence was shown by successive photographs of the same field through the image tube. First, an autophotograph was taken of the luminescence (Fig. 2A) and then a photograph was taken of fluorescence excited by filtered rear illumination (Fig. 2B). In all cases there was an exact correspondence for each site. In the following sections, therefore, it is considered that observations of the fluorescent sites provide a description of the luminescent sites.

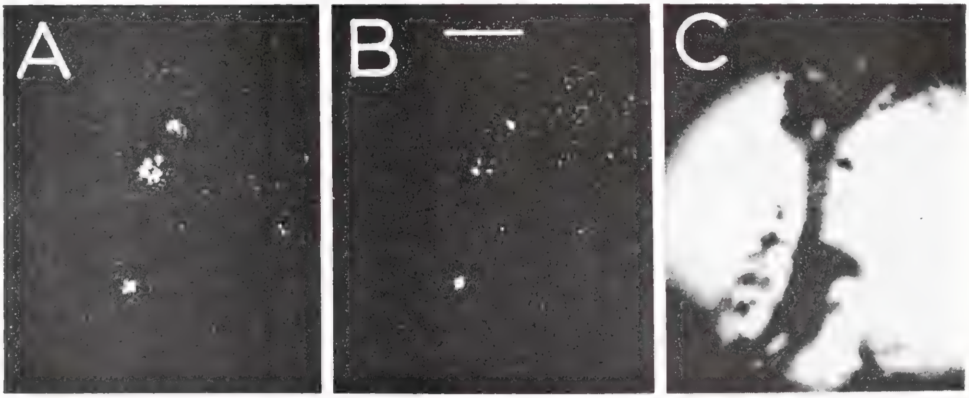

FIGURE 2. Autophotographic (A), fluorescence (B) and rear illumination (C) pictures of an *Obelia* upright. Note the direct correspondence between the luminescent and fluorescent spots (six in each). They directly superimpose. The scale bar indicates 200 μ.

The fluorescence method is extremely useful for characterizing the luminescent sites because the fluorescent emission is not intermittent, as is the luminescence, and the living material can be examined without any manipulation of the colonies (*i.e.*, stimulation, surgical or histological procedures).

Dimensions and shape of the luminescent sites. The size of the luminescent sites as shown by fluorescence varies within the range of 5 to 30 μ with a usual size of about 10 × 20 μ. The sites shown by autophotography are slightly larger than fluorescence sites possibly because of overexposure. The nonfluorescent cells in the same regions as the fluorescent sites have dimensions similar to those of the fluorescent sites. This observation strongly suggests that the luminescent sites are single cells, and we will therefore refer to them as *photocytes*. Histological evidence given below supports this conclusion.

The photocytes possess a wide range of shapes (Fig. 3). They are best observed in very young, distal tissues or in the distal part of the pedicels where the perisarc is relatively transparent and not overgrown by epiphytes (Fig. 3B).

A characteristic feature of these cells is the frequent occurrence of one or

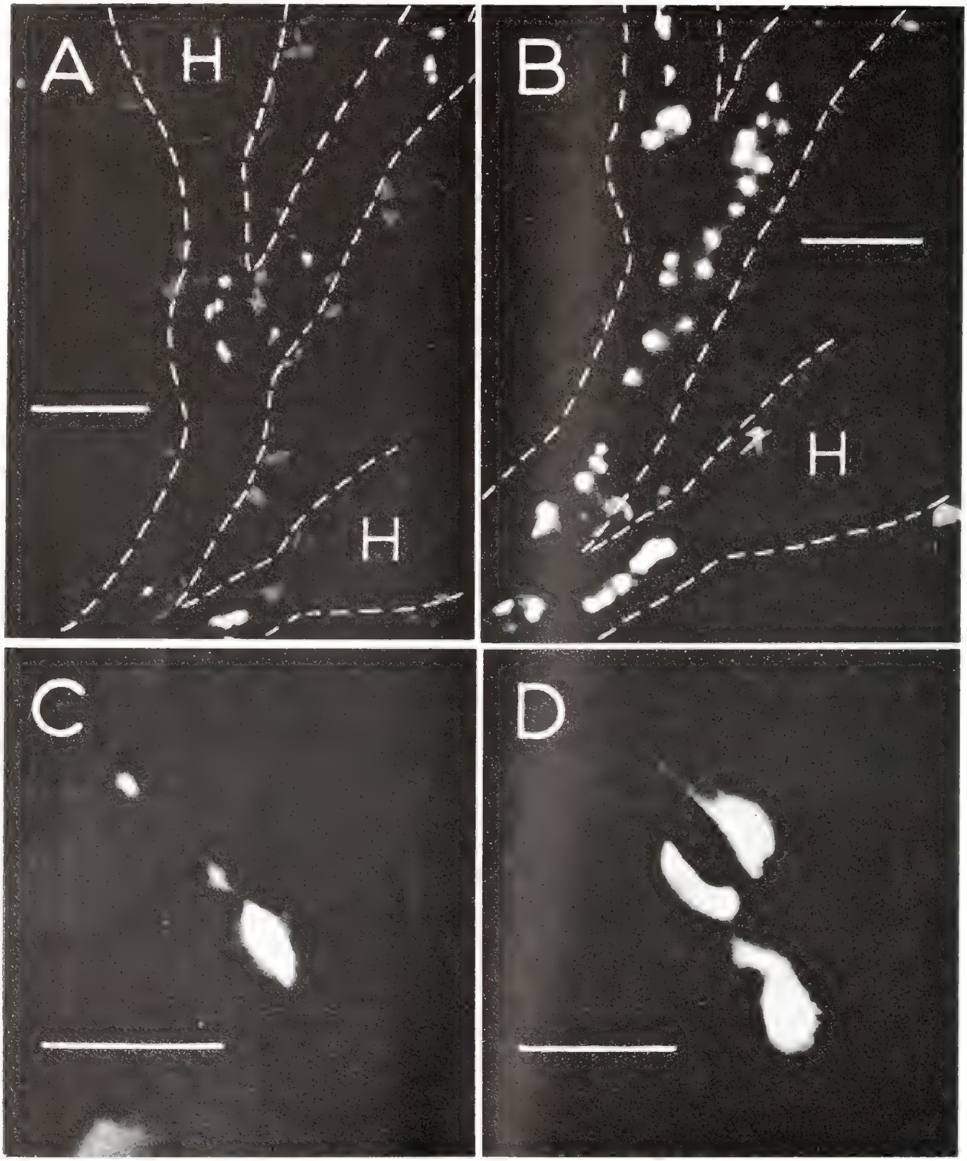

FIGURE 3. Fluorescence microscope photographs of living *Obelia geniculata.* (A). Basal part of an upright showing three nodes; each node and pedicel shows a cluster of photocytes. The skeletal outline of the hydroid has been dotted in white. Hydranths are indicated by an H. The dim fluorescent material around the photocytes are red fluorescing diatoms attached to the hydroid. (B). Distal part of an upright showing two nodes; note the more numerous photocytes than in (A) and the limited diatom growth. Again the outline has been dotted in. (C). Single photocyte in a pedicel; note the long projection. (D). Three photocytes with projections; note the dark presumed nucleus of the upper cell. Bar indicates 40 μ in (A) and (B); 25 μ in (C) and (D).

more cytoplasmic projections that may be up to 20 μ in length (Fig. 3C, D). These projections have no apparent orientation to neighboring photocytes. The distance between photocytes varies from several mm, usually in the stolons or proximal uprights (Fig. 3A), to near contact, especially in the pedicels and distal parts of the uprights (Fig. 3B).

The distribution of fluorescence within a photocyte including the projections usually appears homogeneous in a living whole mount except for a nonfluorescent inclusion (sometimes two) within each cell. The size and location of this inclusion is suggestive of a nucleus (Fig. 3D). Occasionally a compartmentalization of the fluorescence within the photocytes is indicated.

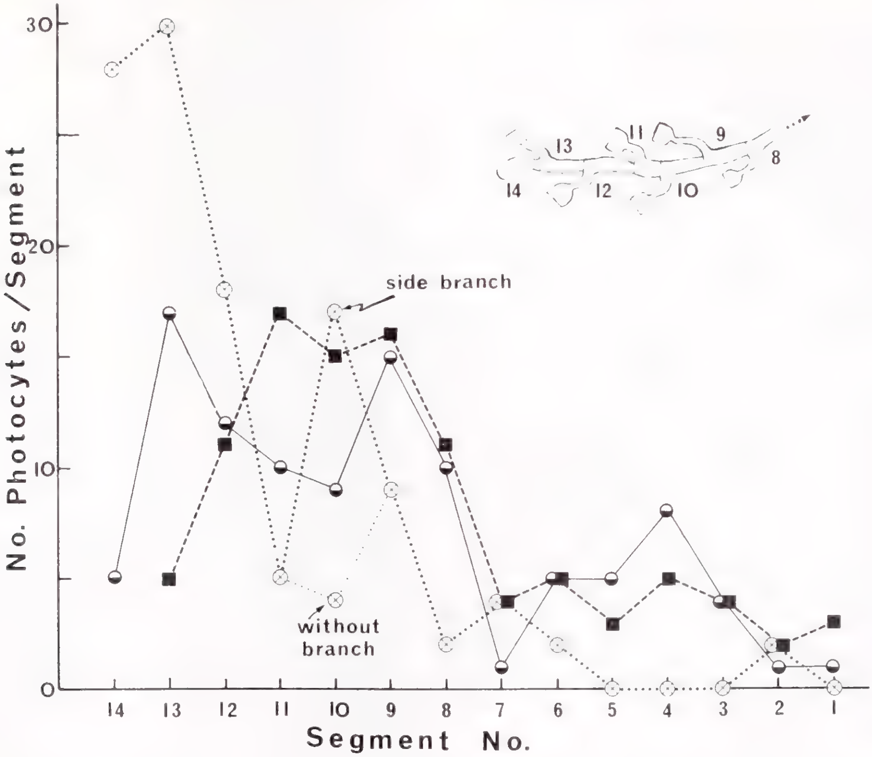

FIGURE 4. The number of photocytes per "segment" (one "segment" as defined here includes the pedicel, node and proximal internode—see diagram, upper right) is plotted against the "segment" number (the oldest, most proximal "segment" is referred to here as the first "segment") for three different, randomly selected uprights. One (circle with a cross) shows the number when a small side branch was and was not considered in the count.

Distribution and density of the photocytes. The general pattern of photocyte distribution and density is shown in the photographs of fluorescence in Figure 3A, B. These photographs show that (1) there are more photocytes in the pedicels and nodes than in the internodes of the upright, (2) the photocytes are all of approximately the same size and (3) there is a greater concentration of photocytes in distal parts of the upright. Also a short side branch is usually marked by a concentration of photocytes, while long side branches show a distribution similar to the primary upright.

The number of photocytes per "segment" (one "segment" as defined here includes a pedicel, node and proximal internode) in uprights from three different colonies is plotted in Figure 4. The variation is considerable, but the generalization that photocytes are more concentrated distally is supported.

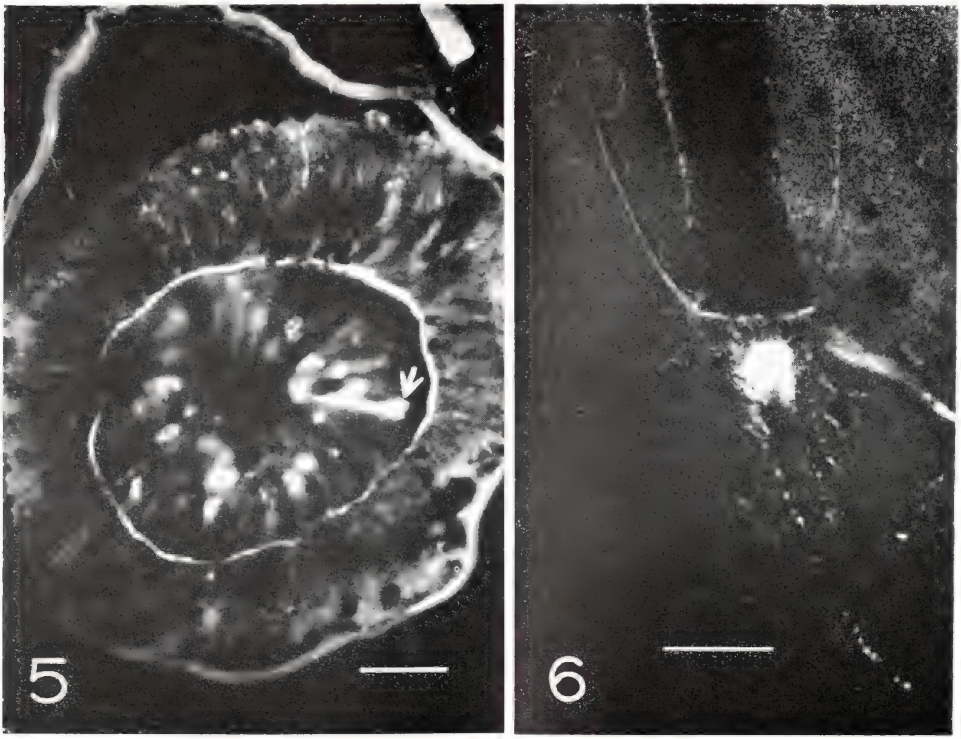

FIGURE 5. Fluorescence microscope photograph of a freeze-dried cross-section of a portion of an upright containing a photocyte (arrow) in the endoderm. Note the long projection of the photocyte toward the coelenteron (central hollow). The endoderm, ectoderm and perisarc are easily distinguished. Scale bar indicates 20 μ.

FIGURE 6. Fluorescence microscope photograph of a living *Obelia bicuspidata* showing the concentration of photocytes at the tip of the pedicel. Bar indicates 100 μ.

Intermittent examination of an upright over a period of several hours showed that the photocytes did not move a visible distance in that time.

Location of photocytes within the endoderm. The observations of living whole mounts indicated that the cells do not lie in the relatively transparent ectoderm (Fig. 3A, B). Fluorescence microscopic examination of freeze-dried, serial sectioned material showed that the photocytes are located in the endoderm (Fig. 5). A reconstruction from serial sections showed a correspondence of the green fluorescent cells seen histologically to the photocyte positions shown in a fluorescence

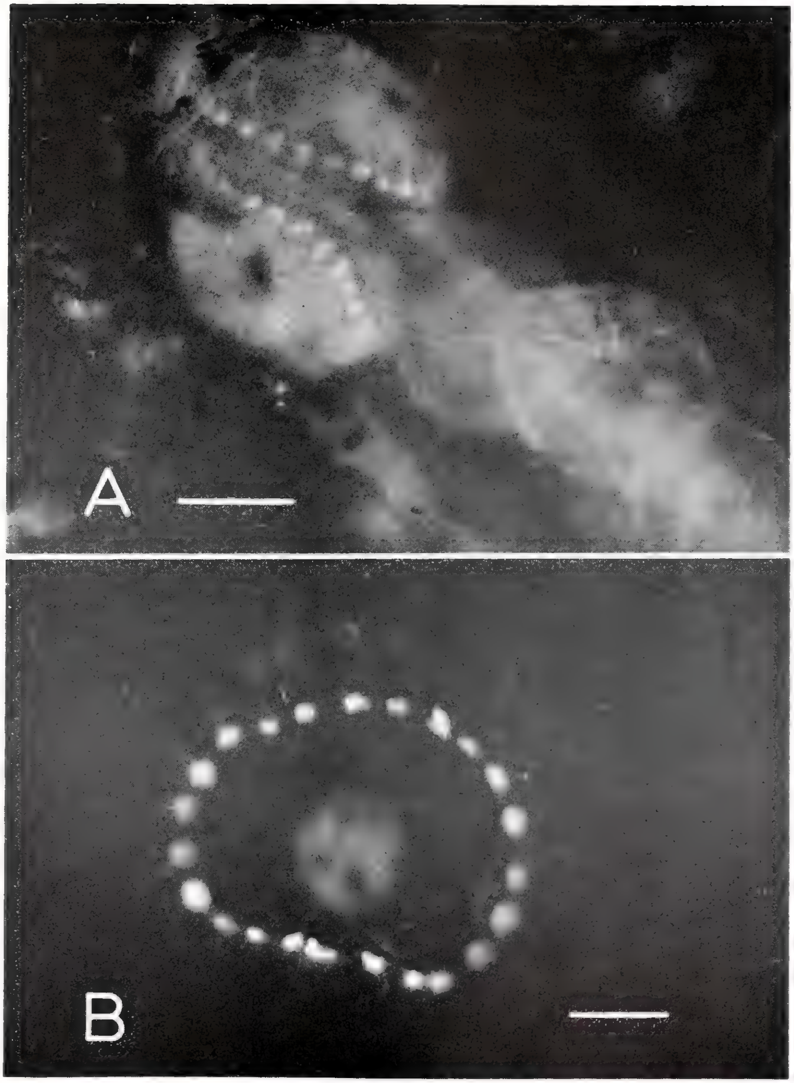

FIGURE 7. (A). Fluorescence microscope photograph of a gonangium containing developing medusae. The most mature medusae (containing the most fluorescence) are toward the left (near the opening of the gonangium). (B). Fluorescence microscope photograph of a newly freed medusa showing the distribution of green fluorescence in the tentacular bulbs; the manubrium shows a yellowish fluorescence and is not luminescent. Scale bars indicate 100 μ. (Living whole mounts).

microscope photograph taken earlier of the same specimen as a living whole mount. Freeze-dried materials showed more fluorescence of the other tissues than did the living material. This greater fluorescence tended to obscure the green fluorescence of the photocytes to a slight degree (Fig. 5).

The bulk of the photocyte, presumably the soma, is usually adjacent to the mesoglea (Fig. 5). Cytoplasmic projections often extend in several directions but not toward the ectoderm. One projection almost always extends to the coelenteron (Fig. 5). Photocytes were never seen in ectodermal tissues. No cases were observed where direct contact occurred between two photocytes. In the freeze-dried sections of photocytes the fluorescent material often appears to be particulate or granular. Since living photocytes appear homogeneous, this granularity may be a fixation artifact.

Fluorescence of the gonangium and the medusa

Although no luminescence following colony stimulation was observed in gonangia in the few cases examined with image intensification (performed prior to the discovery of the fluorescence method), fluorescence microscopy revealed that occasionally photocytes occur within the blastostyle of the gonangium. Visual observations indicated that these photocytes do luminesce upon colony stimulation.

The youngest, developing (proximal) medusae show no fluorescence. More distal medusae show a weak ring of disjunct green fluorescent spots near the ring canal (Fig. 7A). The intensity of the ring of spots, which number sixteen, increases with the age of medusae. Visual observations indicated that these attached medusae do not luminesce upon stimulation of the hydroid colony. Newly released medusae show fluorescence and luminescence at the site of the tentacular bulb at the base of each tentacle (Fig. 7B). As judged from microscopic observations, medusae produce luminescence from these sites upon stimulation with isotonic KCl or direct electric shocks.

The distribution of fluorescent sites is similar to the pattern of luminescence in the hydromedusae *Halistaura* (Davenport and Nicol, 1955).

Distribution of luminescence and green fluorescence among the hydrozoa

It has been previously reported that *Obelia bicuspidata, O. commisuralis, O. longissima, Clytia edwardsi* and *Campanularia calceolifera* showed photometrically recorded flashing responses similar to *O. geniculata* (Morin and Cooke, 1971b). *O. commisuralis* and *C. calceolifera* were examined by means of the image intensifier and proved to have a distribution of luminescence similar to but less dense than that of *O. geniculata.* Fluorescence observations also demonstrated a similar pattern but with fewer photocytes than in *O. geniculata. Clytia edwardsi* shows a greater density of photocytes than in *O. geniculata* by both methods. On the other hand both methods show that the photocytes in *Obelia longissima* and *O. bicuspidata* are concentrated at the tip of the pedicel immediately below the diaphragm of the hydranth (Fig. 6). Using fluorescence methods only, a similar concentrated photocyte distribution was observed in *Lovenella gracilis.*

Several other hydroids were examined photometrically for luminescence using isotonic KCl as the stimulus, and most were also examined for green fluorescence using fluorescence microscopy. In all the cases examined the luminous forms showed green fluorescence while the nonluminous forms showed no such fluorescence (Morin, in preparation). Green fluorescent cells were not found in the hydranths of any species examined. However, a photocyte was observed in the base of the hydranth body in *O. geniculata* in a few cases (less than 0.1% of the hydranths examined).

To date only the families Campanulariidae and Campanuliniidae have been found to possess luminescent hydroid members and not all species within the families are luminescent.

DISCUSSION

The discovery of the fluorescent properties of the photocytes in *Obelia geniculata* has proven to be extremely useful in characterizing the photocyte both structurally and physiologically.

The colonies need not be physically manipulated in any way other than by illumination with a deep blue (460 nm) light in order to see the photocytes. This method, therefore, should be of use to the developmental biologist who is interested in coelenterates, because it allows the study of an effector, dependent on a through-conducting excitation system, which can be monitored at any time without disturbing the colony.

That the fluorescence is maintained within the photocyte even following freeze-drying and embedding is a promising indication that at least mild staining and fixing techniques may be used without destroying the fluorescence and, therefore, that this procedure will ultimately lead to a detailed study of the ultrastructural characteristics of these photocytes.

Titschack (1964, 1966) has also observed this particular luminescent associated green fluorescence in the alcyonarian coelenterates, *Pennatula, Peteroides* and *Veretillum*. We have also reported a similar fluorescence corresponding to luminescence in several hydroids (*Campanularia, Clytia, Obelia, Lovenella, Aequorea* and *Phialidium*) and in the alcyonarians *Renilla, Ptilosarcus* and *Stylatula* (Morin and Reynolds, 1970). The biochemical mechanism responsible for this coupling between the bioluminescence and the green fluorescence have been partially established for a number of hydrozoans and anthozoans (Morin and Hastings, 1971b; Cormier, Hori, Karakanis, Anderson, Wampler, Morin and Hastings, 1973; Wampler, Karkhanis, Morin and Cormier, 1973).

The location of the luminescence in *Obelia geniculata* and all other luminescent hydroids that we have examined is at variance with the conclusions made by Panceri (1876, 1877) on the basis of his study of *Campanularia flexuosa*. He believed that the luminescence resided in the epidermis of all parts of the hydroid including the hydranths. Our results, however, show conclusively that the luminescence is endodermal within only the pedicels, stems and stolons and not in the hydranths. We have performed an approximate duplication of Panceri's experimental procedures of observing a colonly of *Obelia geniculata* after addition of fresh water and find the luminescence too weak for definite visual identification of the cell layer, even with rapid changing from a non-illuminated to an illuminated condition. Furthermore, the tissues began to disintegrate within a few seconds after the fresh water was added. The whole colony became progressively more fluid and disorganized so that anatomical orientation became impossible especially in the hydranth region where the exposure to water first occurs. These observations make Panceri's conclusions open to doubt.

Endodermal localization of bioluminescent cells has also been shown for other coelenterates including the hydromedusan *Aequorea* (Davenport and Nicol, 1955), some pennatulids (Titschack, 1966) and the ctenophore *Mnemiopsis* (Freeman and Reynolds, 1973).

The locations of the bioluminescence in *Obelia* as revealed by autophotography and fluorescence microscopy are curious. Luminescence is located in the least exposed region of the colonies: in the innermost endodermal tissue layer beneath the ectoderm and the skeletal perisarc. The adaptive significance of this peculiar anatomical organization is obscure.

It is interesting to consider the distribution of the photocytes in light of the relative longevity of the tissues. Colonies undergo a regression-replacement cycle in which the hydranth is resorbed into the colony and replaced by a new hydranth. This cycle occurs about once every six or seven days (Crowell, 1961). However, the coenosarc within the pedicels, uprights and stolons does not show such a cycle. During adverse conditions all the hydranths in a colony may regress, and the colony can live for several weeks in this condition. The photocytes are found only in the relatively more stable tissues of the coenosarc, and they are not found in the short lived hydranth tissues. In addition, the density of the photocytes is greatest near the actively growing areas such as the tip of an upright or the site of a side branch.

The photocytes have been shown to be coupled to an excitation system, the luminescent potential (LP) system (Morin and Cooke, 1971b, 1971c). The endodermal position of the photocytes indicates that the LP system is probably also endodermal. It has been shown that another conducting system, the rhythmic potential (RP) system, in *Hydra* is situated within the endoderm (Shibley, 1969).

At present there is no evidence available that shows morphological connections between the photocytes and other cells. The photocytes, at least at the level at which they have been examined, appear to be specialized for a single function: luminescence. It will be of interest to discover whether these cells show multiple functions such as is found in the epitheliomuscle cells of the ectoderm and digestive cells of the endedorm both of which contain myofibrils.

A few generalizations on the systematic distribution of luminescence within the hydroids can be made from our studies that refine those presented by Harvey (1952): (1) There are no known luminescent gymnoblastic hydroids. (2) Only the families Campanulariidae and Campanuliniidae of the Calyptoblastea definitely have luminescent polypoid members. (3) Both these families have luminescent and non-luminescent species. (4) In at least some of the species both the polypoid (hydroid) and medusoid forms are luminescent.

SUMMARY

1. *Obelia geniculata* luminescent sites were examined using image intensifier autophotography and fluorescence microscopy techniques.

2. Autophotography and superimposed fluorescence showed that luminescence emanates from discrete spots located within the pedicels, uprights and stolons of the colonies. The green fluorescent sites correspond to luminescent sites.

3. The size of the spots averages about 10 to 20 μ. They are considered to be cells and are termed *photocytes*.

4. The photocytes are concentrated distally in an upright, are more abundant in pedicels and nodes than in the internodes, and are most abundant in actively growing regions.

5. The photocytes are located in the endoderm against the mesoglea, and they have cytoplasmic projections up to 20 μ long; one of these projections usually extends to the coelenteron.

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ON THE OCCURRENCE OF COLONY SPECIFICITY IN SOME COMPOUND ASCIDIANS[1]

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In some colonial organisms, colony specificity, or histoincompatibility, has been watched recently by many authors. Colony specificity in some compound ascidians is manifested by the fusibility between colonies; two colonies fuse with each other to form a single mass in one case or do not fuse in the other. This problem was first taken up by Bancroft (1903). According to him, two colonies of different origin in *Botryllus schlosseri* do not fuse together after grafting. Fragments from any one single colony, however, easily fuse together. Sister and brother colonies developed from larvae released by one parental colony sometimes fuse and sometimes do not.

Oka and Watanabe (1957, 1960, 1967) encountered similar results in the Japanese ascidian *Botryllus primigenus*, and the genetic control of colony specificity has been further investigated. The colony specificity in *B. schlosseri* also has been investigated, to a lesser extent, by some authors (Sabbadin, 1962; Karakashian and Milkman, 1967).

In *Botrylloides gascoi* and *B. leachi*, according to Bancroft (1903), fusion is invariably established between two colonies, regardless of their origin. Oka and Usui (1944) have reported that for *Polycitor mutabilis*, in which the zooid of a colony exists solitarily in common test, fusion is never seen when two colonies come into contact as a result of natural growth, but when cut surfaces of two pieces are placed in contact, they can be fused to form a single colony. Hence, in these species colony specificity seems to be absent.

Besides ascidians, the occurrence of colony specificity has been known in some groups of coelenterates, *e.g.*, a hydrozoan *Hydractinia echinata* (Hauenshild, 1954, 1956; Ivker, 1966, 1967, 1972; Toth, 1967) and two anthozoans *Eunicella stricta* and *Lophogorgia sarmentosa* (Theodor, 1970). In certain bryozoans (*cf.* Ryland, 1970, page 30) and freshwater sponges (Rasmont, 1970), the presence of colony specificity has also been suggested.

Thus, the colony specificity seems to be a phenomenon widely distributed among colonial organisms. The present paper reveals the presence of colony specificity in some compound ascidians and some insight is given, from the comparative point of view, into the mechanism involved in the establishment of fusion or rejection and, also, into the evolutionary trend of the colony specificity.

MATERIALS AND METHODS

The materials used in the present experiments were living colonies of five compound ascidians, such as *Botryllus primigenus*, *Botrylloides violaceus*, *Pero-*

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[1] Contribution from the Shimoda Marine Biological Station No. 280.

phora orientalis, *Symplegma reptans* and *Didemnum moseleyi*. The first three species were collected in the vicinity of the Shimoda Marine Biological Station, Shizuoka Prefecture, the next two in the vicinity of the Usa Marine Biological Station, Kochi Prefecture.

To facilitate handling of the colonies, they were fixed on glass plates. For *Botryllus* and *Botrylloides*, the colonies taken from nature were fastened to glass plates by the method described by Oka and Usui (1944). For *Perophora*, *Symplegma* and *Didemnum*, the colonies, with the substratum or after separation from it, were fastened to glass plates by tying down the edges with string. The plates with the colonies attached were then set in wooden frames and hung below the surface of the sea. The colonies were thus reared in their natural environment, except for transfer to the laboratory aquarium for performing fusion experiments.

The fusibility of the colonies of *Botryllus, Botrylloides* and *Symplegma,* was tested by fusion experiments. Fusion experiments were routinely carried out by the following procedure: A piece of colony of about 1 sq cm in size was cut out from each of two colonies. The two pieces were placed in juxtaposition on a glass plate and these colony pieces made a contact with each other. The colonies attached successfully on the glass plates after being kept in a moisture chamber in one hour or so, were returned to the culture boxes in the bay as was just mentioned.

For the *Didemnum* colonies, the above method, to attach them on a glass plate in one hour or so, proved to be a not very practical one. Hence, two colony-pieces with contact were fastened to a glass plate by tying them down with string. The plate was then hung in the bay. The string could be removed the following day. For the *Perophora* colonies, use of the following contrivance was made. That is, two stolon-pieces, each consisting of a stolon fragment one centimeter or more in length and some zooids with the actively pulsating heart, were cut out and placed on a filter-paper sheet in a Petri-dish. They were then forced to be in contact at their cut ends. About twenty minutes later filtered sea water was poured in the dish, when the stolon-pieces, now adhered together in a single stolon, floated on the surface. After removal of the filter paper, the stolon-pieces were submerged to the bottom and cultured in the dish. In each species the fusibility of colonies was examined under two conditions, *i.e.,* by bringing either the cut surfaces or their growing edges into contact.

The observation was made one or two days later under a binocular stereomicroscope. In the reaction resulting from contact of two colonies in respective species, three cases can be distinguished. In this paper, by *fusion* is meant the complete union of blood vessels and/or the test matrix of one colony with the same tissues of the other, in other words, the establishment of a common vascular system or a common test between the two. The case in which some active antagonism is seen between the two colonies is referrd to as *rejection* to distinguish from *indifference* in which no particular reaction can be observed.

RESULTS

Botryllus primigenus

Botryllus is a member of the subfamily Botryllinae (Berrill, 1950). In a colony the individual blastozooids are grouped into star-shaped systems and are connected with one another by the ramifying network of vascular vessels, which terminate in ampullae at the periphery of the colony.

The processes of fusion and rejection have already been described and illustrated in detail by Oka and Watanabe (1967) and Oka (1970). Therefore, the brief account of the processes will be described below.

If the cut surfaces of two colonies were brought into contact, either fusion or rejection occurred. In the case of fusion, both the test and the blood vessels of one colony were united with those of the other to form a single colony. In the case of rejection, after about 24 hours the test cells in the contact area became opaque and slightly brown. This change was easily recognizable with the naked eye as a white line in the contact area between the two colonies.

When contact was made between the growing edges of two fusible colonies, ampullae of each colony mutually extended into the test of the facing colony. By 24 hours or so tip-to-side contacts occurred between facing extended ampullae and there fusion took place, *i.e.,* the blood vessels of the two colonies became interconnected. Both by multiplying the number of fused ampullae and by reducing their size to become internal vessels, finally the original two colonies were completely united into a single colony.

It is a singular fact, as has already been pointed out by Bancroft (1903), that the fusion was never seen between the tips of ampullae. It always took place between the tip and the side of ampullae.

When two non-fusible colonies came into contact at their growing edges, the vascular ampullae of each colony actively extended into each other just like in the case of two fusible colonies. Meanwhile, however, a sign of rejection always appeared at the contact area. The first change detectable was that the ampullae and the test cells in the contact area became deep brown and opaque. Then, about two days after contact, the ampullae detached from the body of the colony and finally disintegrated.

Botrylloides violaceus

Botrylloides is also a member of the Botryllinae and has a common vascular system similar to that of *Botryllus.* Bancroft (1903) could not find the evidence of colony specificity in *B. gascoi* and *B. leachi*, but in our species the presence of it has been confirmed.

If two colonies were placed with their cut surfaces in contact, two fusible colonies easily fused together, but non-fusible ones always rejected each other. In the case of rejection, the blood vessels and the test cells of each colony in the contact area turned black in color as in *Botryllus* and disintegrated in about half a day after contact.

When two fusible colonies were allowed to grow naturally towards each other, ampullae of either colony actively extended into the test of the facing colony until they came into contact with the blood vessels. It was often observed that the tips of the ampullae being in contact with the blood vessels became inflated before fusion took place, as if by contact with the blood vessels the normal growth of the ampullae were being obstructed. Fusion took place after the colonies had been in contact for about a day (Fig. 1A). Also in *Botrylloides*, fusion never occurred between the tips of ampullae in each colony.

In contrast to *Botryllus*, two non-fusible colonies took indifference when they came into contact at their growing edges. Ampullae of both colonies bent upwards and remained pushing against each other for several days, as if they were striving to overcome each other. Usually, however, neither the growth of one colony over the other nor the rejection was recognized. Thus, the edges being in contact grew thicker than usual and a deadlock ensued (Fig. 1B). A colony could readily be pulled off by a pair of forceps from the other, showing that no union of the test of each colony was accomplished between the two colonies. When they were separated after a long-term contact, however, occasionally a sign of rejection was found in some test cells of the contact surfaces.

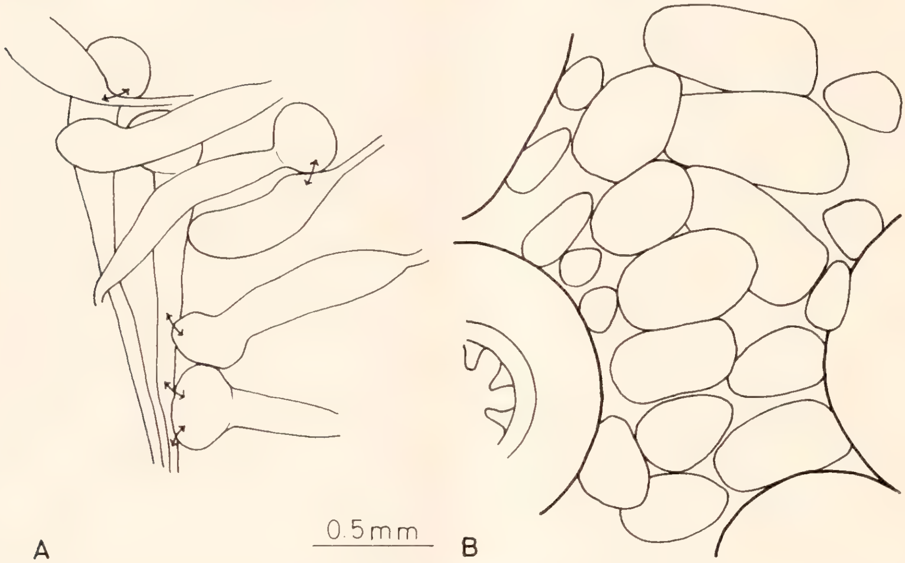

FIGURE 1. Fusion and indifference in *Botrylloides violaceus*. A shows fusion and B shows indifference. In A, arrows indicate the places where the two colonies have fused. In B, two colonies are pushing against each other.

Symplegma reptans

Symplegma also belongs to the Botryllinae and has a common vascular system similar to that of *Botryllus*. Among the colonies of *S. reptans*, the presence of colony specificity has been revealed.

When there was contact between the cut surfaces of colonies, either complete fusion or complete rejection took place. The processes of fusion and rejection were essentially similar to those observed in *Botryllus* and *Botrylloides*. In the case of rejection, the blood vessels and the test cells in the contact area gave a necrotic appearance.

When the growing edges of two fusible colonies came into contact, they pushed against each other. In most cases, the ampullae of both colonies remained being in contact at their tips for about a day (Fig. 2A) and finally fusion took place between them (Fig. 2B). As is clear in Figure 2B, in this species tip-to-tip fusion of ampullae was rather common, though of course tip-to-side fusion was also observed in some cases.

In *Symplegma* the ampullae seem to be associated with one another too closely to allow the invasion of the ampullae of another colony. While, in *Botryllus* and *Botrylloides*, in which tip-to-side fusion is usual, the association seems to be too loose to prevent the invasion of the ampullae of other colony. This will be the reason why the tip-to-tip fusion occurs only in *Symplegma*.

When the growing edges of non-fusible colonies came into contact, usually the ampullae of both colonies were pressed tightly against each other as in the case of fusion. About one or two days later, however, the distal parts of ampullae became dark and opaque. Then, the ampullae retracted almost invariably away from the contact area, leaving there the empty test in which they had formerly extended. In most cases, the boundary of the two colonies was clearly seen under a binocular stereomicroscope. A stage of this rejection is illustrated in Figure 2C.

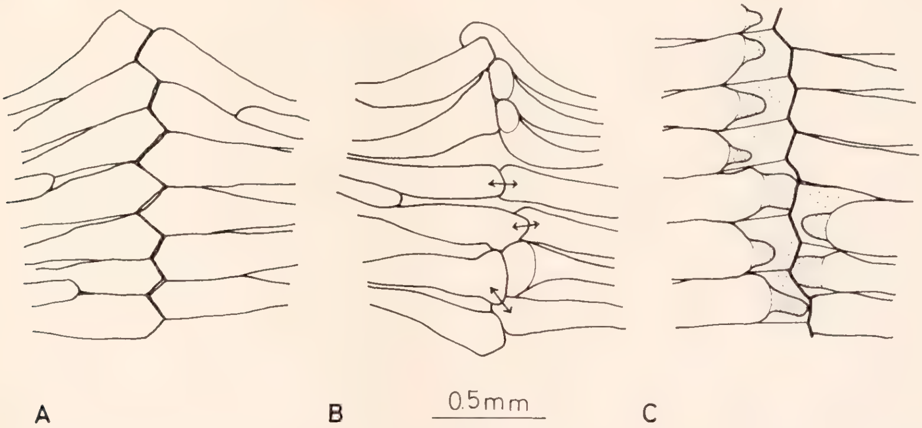

FIGURE 2. Fusion and rejection in *Symplegma reptans*. In A, two colonies are in contact at their growing edges. B shows fusion; fused ampullae are indicated by arrows. C shows rejection. Distal parts of ampullae (densely dotted) have been retracted leaving the empty test (sparsely dotted). The boundary between the two colonies is clearly detected.

Before the sign of rejection appeared, each colony being in contact could be easily separated by a pair of forceps from the other. Once it was observed, however, they were no longer separable at the boundary line, thus indicating that the retraction of ampullae was brought about after the establishment of some union between the test matrices of both colonies.

In the process of rejection, the breakdown of ampullae subsequent to their penetration into the test matrix of the other colony, which was the case in *Botryllus*, never occurred in *Symplegma*. In the colonies in which rejection took place, new ampullae always budded at the basal part of the retracted ampullae and continued to grow over them.

The process of rejection presented above is applicable to the combination of colonies of equal strength, armed with the same weapons. If the growing edges of non-fusible colonies, one begin considerably thicker and containing more ampullae than the other, came into contact, the thinner edge was pushed over by the thicker edge instead of being opposed to it. Thus, indifference ensued without giving any evidence of rejection between them. In general, new ampullae just regenerated from the cut ends of blood vessels were less vigorous than the old ones.

Didemnum moseleyi

Didemnum belongs to the family Didemnidae; the colony has no common vascular system in contrast to the above three species. Each zooid is being embedded solitarily in common test and has some vascular processes with faint circulation of blood, which terminate in enlarged bulbs or ampullae. The test usually contains numerous bladder cells, being characteristic of this family. In *Didemnum* also, the presence of colony specificity has been revealed.

If we cut out two pieces from a colony and placed them with their cut surfaces in contact, they fused completely to form a single colony. About 24 hours after contact, the boundary between the original two pieces could not be detected.

On the other hand, rejection resulted from contact between the cut surfaces of two pieces derived from different colonies. The following day after operation, numerous bladder cells existing in the contact area showed an opaque appearance, and the vascular processes had retracted away from that area. Though a union had been established between the test matrices of the two pieces, the boundary line between them could still be detected. Then, at last, the contact area collapsed and disintegrated. Only five colonies were at our disposal, all of which were mutually non-fusible.

When two pieces derived from a single colony came into contact as a result of natural growth, they extended their vascular processes into each other. Thus, at first the two pieces were connected with a thin sheet of test containing vascular processes and later zooids appeared in that area. In the course of this fusion, union between vascular processes never took place.

The pieces derived from different colonies rejected each other when also the contact was made between their normal edges. In the actively growing edges of the colony, vascular ampullae were crowded. When two such edges being non-fusible were placed in apposition, most of the ampullae of each colony always retracted away from the surface shortly before they came into contact, thus giving the impression that the ampullae deprived the colony of its vigor from that area. Some of the ampullae of each colony, however, continued to grow and extended towards each other. After contact, the ampullae and their surrounding test became opaque and finally disintegrated. Two stages of this rejection are illustrated in Figures 3A and 3B.

Perophora orientalis

Perophora belongs to the family Perophoridae, in which respective zooids are connected with one another only by basal stolonic vessels from which they have arisen as buds. The stolons are sometimes branched, but not interconnecting the individuals in a complex vascular network as in *Botryllus*.

From contact between the normal surfaces of two stolons, indifference always ensued regardless of their origin. That is, they continued to grow, adjoining together side by side or crossing one over the other.

When two stolon-pieces cut out from a colony were forced to come into contact closely at their cut ends, they easily fused, *i.e.*, the blood could be distinctly recognized passing from one piece to the other. The same occurred even when two pieces derived from different colonies were used. We used five colonies in all; all of the five colonies were mutually fusible. Thus, two stolons, not only of the same colony but also of differnt colonies, could be easily fused together by grafting. From these results, the conclusion seems to be justified that in a *Perophora* colony specificity is absent.

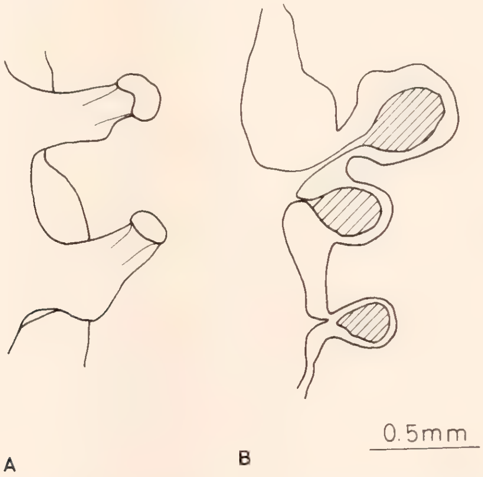

FIGURE 3. Rejection in *Didemnum moseleyi*. In A, two vascular processes of the left-hand colony are extended on the right-hand colony. In B, disintegration occurred in the vascular processes and their surrounding test.

Heterogenetic combinations

Using respective colonies of three species, *Botryllus*, *Botrylloides* and *Symplegma* belonging to the Botryllinae mentioned before, heterogenetic or interspecific combinations were prepared.

When the cut surfaces of the two colonies of different species were brought into contact, no special phenomenon could be observed between them. In the same way, when the two colonies of different species were allowed to grow towards each other, neither fusion nor rejection was seen to occur between them. They simply competed with each other for the substratum to grow upon. Thus, contact between the two colonies of different species always resulted in indifference.

DISCUSSION

The results from the present experiments, together with those of Oka and Usui (1944) on *Polycitor* colonies, are summarized in Table I. In the reaction resulting from contact between two colonies, fusion, rejection and indifference are distinguishable. Of these, fusion and rejection are essential; indifference is simply a defect of the other two.

Colony specificity can be considered as a type of allogeneic recognition and manifests itself among others as a hindrance to fusion between two colonies. As is clear from Table I, colony specificity is present in some species, such as *Botryllus, Botrylloides, Symplegma* and *Didemnum*, but not in *Perophora* and *Polycitor*. According to Bancroft (1903), *Botrylloides gascoi* and *B. leachi* colony specificity is absent, *i.e.*, a union of two colonies is always established either by grafting or by natural growth, regardless of their origin. Discrepancy between the results obtained by Bancroft and the present experiments will be due to the limited number of his observations rather than to variance of species.

TABLE I

Summarizing representation of the results of fusion experiments

Species	Contact between cut surfaces	Contact between growing edges
Botryllus primigenus	Fusion Rejection	Fusion Rejection
Botrylloides violaceus	Fusion Rejection	Fusion Indifference
Symplegma reptans	Fusion Rejection	Fusion Rejection (Indifference)**
Didemnum moseleyi	Fusion Rejection	Fusion Rejection
Perophora orientalis	Fusion	Indifference
*Polycitor mutabilis**	Fusion	Indifference
Heterogenetic combinations	Indifference	Indifference

* Oka and Usui (1944).

** When one very vigorous and one rather weak edge come into contact, sometimes indifference ensues.

In those species in which the presence of colony specificity is assured, the growing edges of the same colony fuse together with the complete union of test and/or blood vessels whenever they come into contact. On the other hand, in those species in which colony specificity is absent, when the growing edges or the test surfaces of the same colony are brought into contact, indifference can be recognized. In summary, the presence or absence of colony specificity may be correlated with the ability or inability of fusion between the growing edges of the same colony. Judging from these facts, colony specificity seems to be a feature of common occurrence in those ascidians in which fusion of test and/or blood vessels always occurs between the growing edges of its own. The validity of this assumption in other colonial organisms should be examined by future researches.

When two colonies of different species are brought into either by natural growth or by experimental means, they show indifference to each other. Thus, as has been pointed out by Oka (1970), fusion or rejection recognizable in ascidians is a feature specific within the species, though in certain cnidarians, heterogenetic rejection has been reported (Katô, Hirai and Kakinuma, 1967; Theodor, 1970).

As has been suggested by Oka and Usui (1944) with *Policitor* colonies, the test of ascidians seems to consist at least of two layers, one external and the other internal, the former being thin and tough as compared with the latter. Taking the process of fusion or rejection between the growing edges into consideration, at least two steps will be separated. The first step is the elimination of the external layer of the test, which will be attained enzymatically. The second step is the reaction leading to the completion of fusion or rejection, which itself may consist of a series of reactions.

The first step is either possible or impossible, being alternative. In the second step, however, it seems that the two processes or mechanisms of fusion and rejection are not necessarily alternative; in a sense the former will be more basic and may be overlapped by the latter. In the case of rejection at the growing edges of *Botryllus*, for instance, the two colonies being in contact extend their ampullae into each other as in the case of fusion; before the union of vascular systems takes place, however, the ampullae begin to disintegrate. Judging from these facts, it will be the case that the process of fusion is being interrupted by that of rejection, if it is present. The former is completed only when the latter is absent.

Provided that both the first and the second steps can proceed after contact of tissues, which seems to be the case at least in the botryllids, the following will be justified: In the contact between growing edges, if the first step is lacking the second step is not realized, when indifference ensues. On the contrary, in the contact between cut surfaces the first step is experimentally attained, when either fusion or rejection occurs.

On the basis of the above discussion, the results obtained with respective species will be interpreted below.

In *Polycitor mutabilis* and *Perophora orientalis*, in which colony specificity is absent, the mechanism of the elimination of the external layer is completely lacking. Accordingly, when there is contact between the test surfaces indifference ensues. When there is contact between the cut surfaces, however, fusion occurs, because they are naked, *i.e.*, there is no layer between them.

In *Botryllus primigenus* the elimination of the external layer is always complete when two colonies come into contact at their growing edges, without reference to their relation, fusible or non-fusible. Therefore, in this species the first step is not colony specific. Colony specificity exists only in the second step, and either fusion or rejection is attained.

The results obtained in *Didemnum moseleyi* are similar, seemingly at least, to those of *B. primigenus*. In *Didemnum*, however, most of the vascular ampullae of non-fusible colonies retract away from the surface shortly before they come into direct contact. Only some of them grow upon the other colony and undergo disintegration. These results may be taken to indicate that in this species the substances participating in rejection can pass, to some extent at least, out of the colony. No definite conclusion, however, can be drawn, until suitable studies are carried out.

Botrylloides violaceus will be worthy of particular notice. In this species rejection occurs only when contact is made between the cut surfaces of non-fusible colonies; indifference is always obtained when they come into contact at their growing edges. In contrast to that, when fusible colonies are brought into contact either by means of natural growth or by grafting, fusion invariably takes place Thus, in this species both the first and the second step will be considered as colony specific. It should be noticed here that in *B. violaceus* the specificity of the first step is completely correlated with that of the second step, in other words, fusion and indifference between growing edges perfectly correspond to fusion and rejection occurring between cut surfaces, respectively. How this correlation is governed is an open question.

In *Symplegma reptans* rejection between growing edges takes place only when two colonies remain pushing against each other for a definite period. In this process neither the penetration of ampullae of one colony into the other nor the extinction of the boundary between them is observed. Once any sign of rejection is seen, however, the two colonies cannot be artificially separated from each other indicating the establishment of some union between the test matrices. Since observations on this species are limited, we cannot say indiscreetly whether the elimination of the external layer of the test, *i.e.*, the first step of rejection, is brought about by means of chemical action which may be the case with *Botryllus,* or by means of physical action as a result of pushing against each other. Provided that the latter is the case, both the first and the second step will be colony specific.

More recently, the significance of the colony specificity in relation to the evolution of adaptive immunity of vertebrates has been discussed by Burnet (1971). On the basis of the above discussion and from the view point of the evolution of colony specificity, the colonial organisms may be classified into the following four groups. (1) Colonies have neither the specificity nor the structural barrier to fusion. Some sponges may belong to this group. (2) An external layer which prevents fusion with one another is formed, but the specificity is still lacking. In this group, to which *Perophora* and *Polycitor* belong, the individuality is assured simply by the structural barrier. (3) Specificity in the second step of fusion is acquired. In those species in which the substances participating in rejection can pass through the external layer of the colony or in which no such a layer is differentiated, rejection may take place without colonies coming into direct contact. Such species also, if they exist, should be classified in this group. Thus, *Botryllus* and *Didemnum* belong to this group. (4) Colony specificity is established in both the first and the second steps of fusion. To this group *Botrylloides* and *Symplegma* (?) belong.

It is our present duty to acknowledge our indebtedness to the staff of the Shimoda Marine Biological Station or the Usa Marine Biological Station.

SUMMARY

1. The presence or absence of colony specificity, *i.e.*, the recognition of self and not-self in colonial organisms, has been investigated with several species of compound Ascidians. If the reaction resulted from contact either between growing edges or between cut surfaces of colonies, fusion, rejection and indifference have been distinguished. Of these three cases, indifference means simply a defect of the other two. Both fusion and rejection are specific within the species.

2. The presence of colony specificity has been demonstrated in *Botryllus*

primigenus, Botrylloides violaceus, Symplegma reptans and *Didemnum moseleyi.* But, in *Perophora orientalis* colony specificity is absent.

3. From the above facts, it has been suggested that the colony specificity may be a feature being common to those ascidians in which fusion of test and/or blood vessels always occurs between the growing edges of its own.

4. In the process of fusion or rejection at the growing edges, two steps have been distinguished. The first step is the elimination of the external layer of test, which is followed by the second step terminating in the completion of fusion or rejection. In *Botryllus* and *Didemnum,* only the second step is colony specific. In *Botrylloides,* however, both the first and the second step are colony specific.

5. A possible evolutionary trend of colonial organisms in relation to the colony specificity has been represented.

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LOCOMOTOR ACTIVITY RHYTHMS OF JUVENILE ATLANTIC SALMON (*SALMO SALAR*) IN VARIOUS LIGHT CONDITIONS

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Endogenous circadian components in the activity rhythms of fishes have not been convincingly demonstrated. Various circadian oscillations, subject to phase setting primarily by natural or artificial light cycles, have been documented in several fishes. This synchronizing effect of light cycles on the locomotor activity patterns of salmonids, in particular, has been cited in several instances (Ali, 1964; Swift, 1964; Byrne, 1968; Varanelli and McCleave, 1974).

Whereas circadian rhythms in certain mammals and birds have been known to free-run in constant conditions for months at a time (Aschoff, 1960, 1966), the rhythms termed "endogenous" in various fish studies have usually not been apparent for more than a few days. Activity rhythms have persisted for two or three days in constant conditions in juvenile Atlantic herring, *Clupea harengus*, (Stickney, 1972), juvenile sockeye salmon, *Oncorhynchus nerka*, (Byrne, 1968), juvenile Atlantic salmon, *Salmo salar*, (Ali, 1964), and in a European minnow, *Leucaspius delineatus*, (Seigmund and Wolff, 1973). The adult sea lamprey, *Petromyzon marinus*, maintained a circadian activity rhythm for five days in constant dim light at which time observations were discontinued (Kleerekoper, Taylor and Wilton, 1961).

An exceptional case is the circadian rhythm of the swell shark, *Cephaloscyllium ventriosum*, which continued for about 15 days in both constant light and constant darkness (Nelson and Johnson, 1970). The same authors did not observe free-running rhythmicity in the horn shark, *Heterodontus francisci*.

Circadian rhythms of swimming speed in the bluefish, *Pomatomus saltatrix*, have been reported to dissipate after two or three days in constant dim light, but later to become reestablished (Olla and Studholme, 1972). A similar reestablishment of rhythmicity has been suggested for Atlantic salmon (Ali, 1964).

Gibson (1971) found that exposure to light-dark cycles was necessary for as long as two to four months before circadian rhythms which would persist in constant darkness could be entrained in blennies, *Blennius pholis*. Activity rhythms with a 12 hr period were easily entrained in the same fish by changes in hydrostatic pressure resulting from local tidal cycles.

A circadian rhythm of electric organ discharge has been observed to free-run in constant dim light in the electric gymnotid, *Gymnorhamphichthys hypostomous*, (Lissmann and Schwassmann, 1965). The appearance of the corresponding locomotor activity rhythm in these fish seems dependent upon the presence of a light-dark cycle and the natural environmental substrate. It was concluded that the activity rhythm is not a good indicator of the endogenous oscillation (Schwassmann, 1971).

Locomotor activity patterns in juvenile Atlantic salmon were observed by

Varanelli and McCleave (1974). Photoperiod and temperature were altered in different experiments to approximate concurrent seasonal conditions. Activity rhythms became synchronized to the imposed light-dark cycle, but in most cases failed to persist in constant conditions.

The present study was designed to gain more information about the effects of light regimes on the activity patterns of Atlantic salmon by subjecting larger numbers of fish to the same experimental conditions. About two thirds of the fish were simultaneously tested for their responses to weak extremely low frequency electric and magnetic fields. Since these fields were found to have no effect on activity (Richardson, McCleave and Albert, unpublished data), fish exposed to the fields are combined with control fish in this paper.

MATERIALS AND METHODS

Between June 1972 and June 1973, 16 experiments involving 192 fish were carried out. Usable records were obtained from 177 fish. In each experiment the locomotor activity of 12 fish was individually recorded for 10 days, though a few fish records were less than 10 days due to equipment failures. The fish were exposed to a 12 hr light–12 hr dark cycle (LD 12:12) in eight experiments, to constant light (LL) in four experiments, and to constant darkness (DD) in three. In one experiment, a 23 hr cycle containing 1 hr of light (LD 1:22) was imposed.

Sea run Atlantic salmon parr between 13 and 20 cm total length were obtained from the Craig Brook National Fish Hatchery in East Orland, Maine. Fish were placed in individual activity chambers as soon as they arrived at the university and were allowed two days to acclimate to the apparatus.

The apparatus and recording methods were similar to those described by Varanelli and McCleave (1974). The activity chambers were circular channels 10 cm wide by 27 cm deep, built from two concentric polyethylene cylinders, 47 cm and 27 cm in diameter. Air was bubbled into the center of the inner cylinder and diffused into the channel through holes in its inner side. Two activity chambers were housed in each of six light-tight water baths. The chambers were water-tight and opaque so that fish in the same water bath had no visual or chemical contact with one another. Water was circulated through the baths from a refrigeration unit which maintained the temperature at 15 ± 1° C. Temperature was continuously recorded in one of the chambers.

Activity was recorded electromechanically. Two plastic probes were suspended in opposite sides of the swim channel. The ends of the probes hung 4 cm off the bottom. The upper portion of each probe was attached to a piece of copper braid which hung through a carbon ring. When a fish moved the probe, contact was made with the carbon ring completing an electric circuit. This caused one count to be registered by an automatic counter. The two probes from each chamber were wired in parallel to one counter, which printed 10 times per hour. Counter tapes were removed once each day and the activity counts were coded directly for computer processing.

Fish were not fed during the experiments to eliminate inducement of activity cycles based on feeding. Experiments were carried out in a basement laboratory which was entered only once each day at 1200 EDT. Efforts were made to isolate the fish from as many environmental disturbances as possible.

Light was provided by either fluorescent or incandescent bulbs mounted on the lid of each water bath. Illumination ranged between 1 and 15 lux at the water surface depending on the area of the tank measured. In the LD 12:12 experiments, automatic timers switched the lights on at 0600 EDT and off at 1800 EDT each day.

Computer drawn plots of the hourly activity for each fish over the course of each day were obtained. Composite plots encompassing all 10 days were also produced for each fish. These plots were visually examined to determine the nature of individual activity patterns. Data from fish with similar patterns were combined to produce plots illustrating typical patterns.

TABLE I

Summary of locomotor activity patterns and periodicities of Atlantic salmon parr in LD 12:12

Experiment date	Number of salmon				Range of period lengths	Mean period length
	Diurnal	Nocturnal	Light change	Aperiodic		
6/22–7/ 2/72	11	0	1	0	23.8–24.7	24.1
7/ 7–7/17/72	5	0	6	0	23.9–24.0	24.0
7/21–7/31/72	6	0	4	0	24.0–24.2	24.1
8/ 4–8/14/72	8	1	0	0	24.0	24.0
2/15–2/25/73	1	8	3	0	24.0–24.5	24.1
3/ 3–3/13/73	3	4	4	1	24.0–24.5	24.0
4/15–4/25/73	1	5	5	0	23.9–24.6	24.1
5/16–5/26/73	0	2	9	0	23.9–24.0	24.0

Activity records were analyzed for periodicity by the periodogram method of Enright (1965). This method is appropriate for serially correlated data because it does not require random independent observations. The periodogram method calculates amplitudes for a series of possible period lengths within the range of interest. The amplitude is the standard deviation of the hourly means of activity. The hourly means used for each amplitude were drawn from arrangement of the data based on the assumption that a given period existed. If a true periodicity exists the amplitude produced by the proper assumed period length will be clearly larger than the other amplitudes. Computer calculated amplitudes were obtained for assumed period lengths between 3.0 and 33.0 hr in increments of 0.1 hr.

Results

LD 12 : 12

Periodogram analysis showed that fish subjected to a 12:12 light-dark cycle possessed a rhythmicity in locomotor activity with a period length very close to 24.0 hr (23.8–24.7) (Table I, Fig. 1a). Only one fish in 88 was aperiodic. All periodic fish exhibited one of three typical patterns: diurnal, nocturnal, or active primarily at times of light-dark transitions. From all eight LD experiments

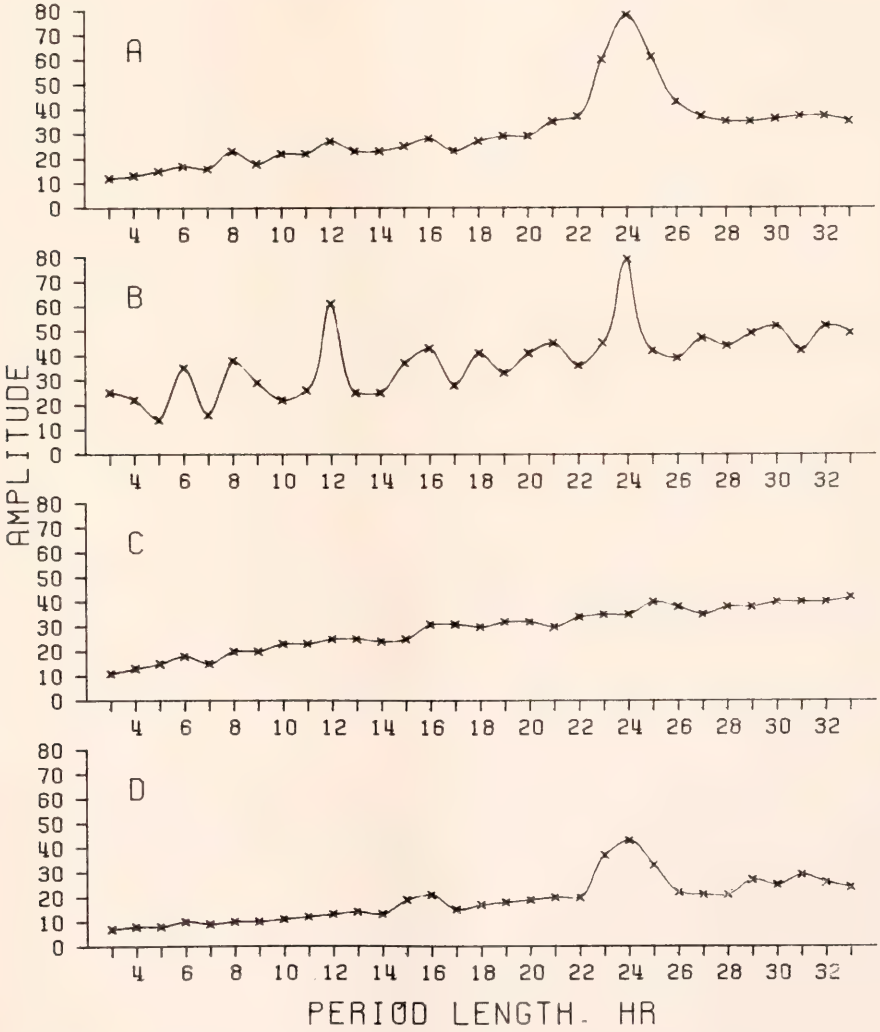

FIGURE 1. Sample periodograms for (a) a diurnal Atlantic salmon in LD 12:12, (b) a light-change-active salmon in LD 12:12, (c) an aperiodic salmon in DD, (d) salmon in DD with 24.0 hr periodicity. (Periodograms were calculated in steps of 0.1 hr, but only hourly points are plotted for clarity).

35 fish were diurnal, 20 were nocturnal and 32 were active at light change (Fig. 2). Mean percentages of daily activity occurring when lights were on ranged from 53 to 94% for diurnal fish, when lights were off from 62 to 86% for nocturnal fish, and in the 3 hr following lights on and lights off from 28 to 55% for light-change-active fish (Fig. 3). Expected percentages, if activity were equally distributed throughout the day, were 50%, 50%, and 25%, respectively, for diurnal fish, nocturnal fish, and light-change-active fish.

In all three patterns the most abrupt increase in hourly activity occurred during

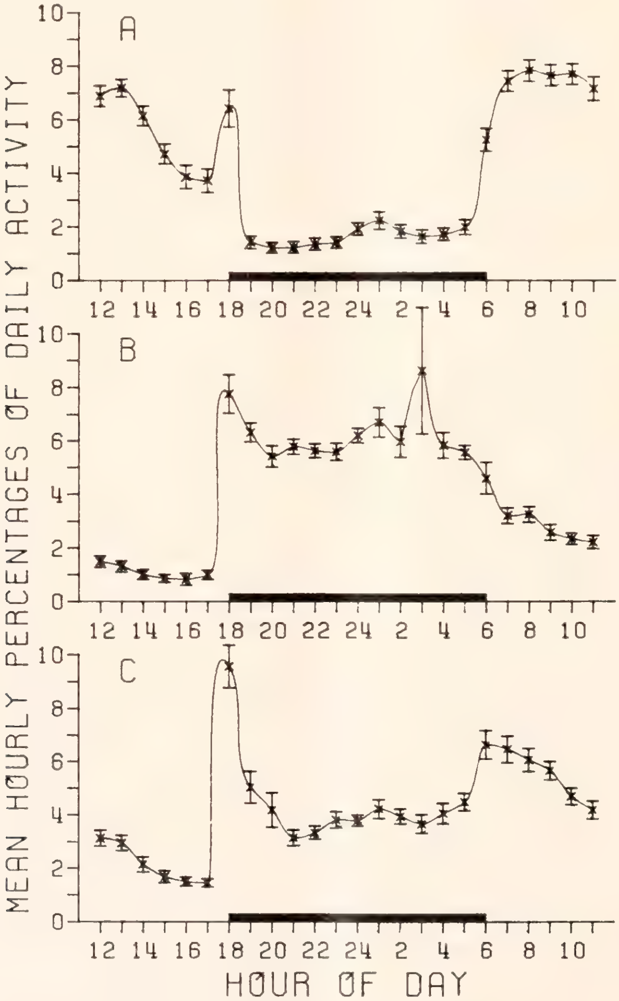

FIGURE 2. Composite plots of mean hourly percentages of daily activity ± 1 standard error for Atlantic salmon in LD 12:12 showing the three activity patterns; (a) light-active fish, N = 35, (b) dark-active fish, N = 20, (c) light-change-active fish, N = 32. Dark bar indicates lights off.

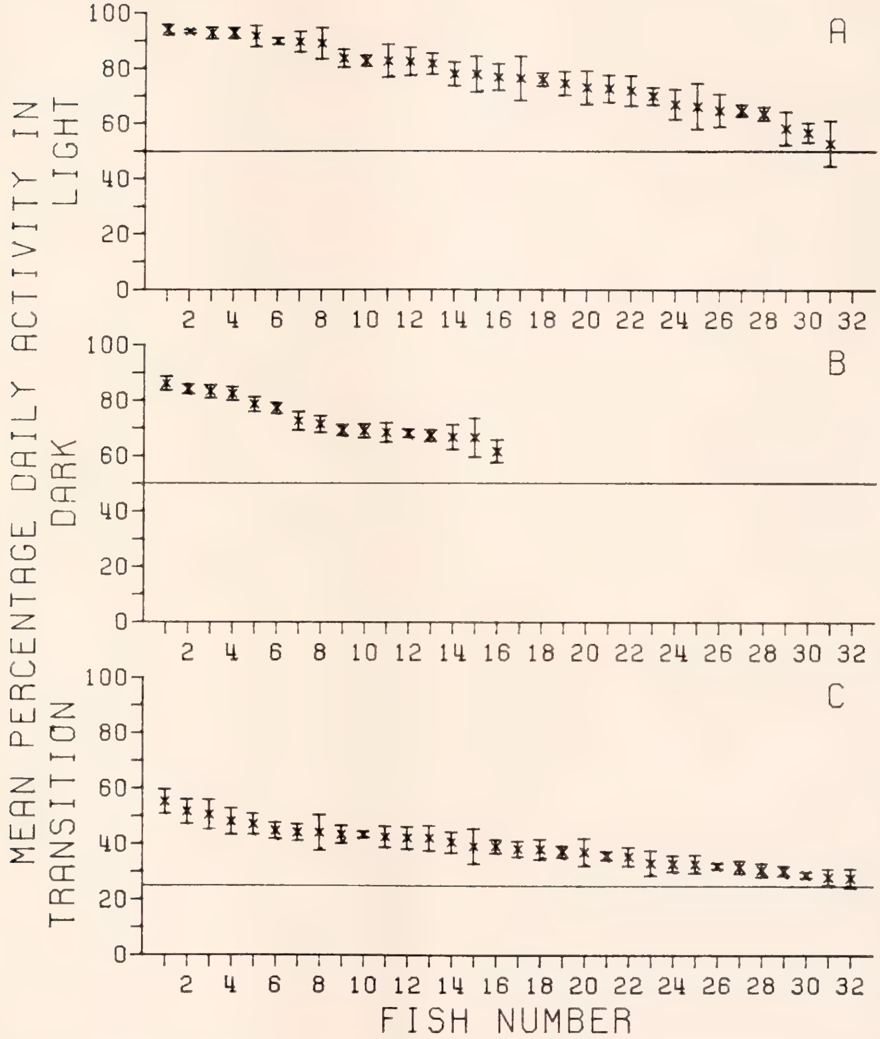

FIGURE 3. Mean percentage of daily activity ± 1 standard error in LD 12:12 (a) of diurnal Atlantic salmon when lights were on, (b) of nocturnal salmon when lights were off, (c) of light-change-active salmon in the 3 hr following lights on and lights off. Only those salmon for which a full 10 day record was available were used.

the hour immediately following lights off (Fig. 2). Diurnal and light-change-active fish showed a second burst of activity in the hour following lights on. Nocturnal fish showed a less abrupt response to lights on in the form of decreasing activity over several hours.

Most light-change-active fish (30 of 32) exhibited a pattern very similar to that depicted by the composite plot (Fig. 2c). The least amount of activity occurred during the 6 hr preceding lights off. An abrupt rise at lights off was followed by an intermediate level of activity during the dark interval. A second burst at lights on was followed by a higher level of activity for the next 6 hr. The other two fish in this group had high levels of activity only at times of light change and were relatively inactive at other times. Two fish showed some evidence of anticipating lights on by an increase in activity during the 2 hr preceding the transition.

The periodogram of light-change fish contained distinct secondary maxima at the period length of 12.0 hr (Fig. 1b). The periodograms of nocturnal or diurnal fish occasionally showed secondary peaks which were less distinct. Some diurnal fish showed recurring bursts of activity shortly after midnight which lasted for one or two hours.

Four LD experiments were carried out during the summer of 1972. Most fish (30 of 42) were diurnal (Table I). Eleven fish were light-change-active and only one was nocturnal. In contrast, Atlantic salmon tested during the previous summer in LD cycles of length approximating the natural cycle were about equally distributed among the three activity patterns (Varanelli and McCleave, 1974). Four additional LD experiments of the present study took place during the winter and spring of 1973. In these experiments 19 of 46 fish were nocturnal, 21 were light-change-active and five were diurnal. The trend was from mostly nocturnal activity in the winter to more light-change activity in the spring.

Constant conditions

Fish subjected to LL or DD were similar in being generally aperiodic (Fig. 1c). Intermediate levels of activity were distributed fairly equally over the day in the form of sporadic bursts. Five fish (of 30) in DD produced periodograms which had peak amplitudes at period values near 24.0 hr (23.5–24.2 hr) (Fig. 1d). In only two of these cases could a pattern be found by visual inspection of the daily plots. These two series of plots suggested recurring peaks near subjective evening. No fish in LL showed any evidence of periodicity.

The mean of the total activity counts was computed for each group of experimental fish. The mean from the LL experiments (36,670) was about twice as large as the mean from DD (17,240). The mean from LL experiments was close to the LD mean (31,100). The highest mean total activity (45,000) came from fish subjected to 1 hr of light (LD 1:22).

Short signal entrainment

Twelve fish were exposed to a 1 hr light stimulus beginning every 23 hr for 10 days in an attempt to initiate a 23 hr rhythmicity of activity. Most fish (11 of 12) were aperiodic. In one case a maximum amplitude occurred at 24.0 hr, but no pattern was obvious from the plotted data.

DISCUSSION

Locomotor activity patterns of juvenile Atlantic salmon were entrained by an artificial light-dark cycle. The fact that about one third of the fish had activity induced at times of light change, while the other two thirds were divided between dark-active and light-active suggests that the transition stimulus rather than the light intensity may be of primary importance in synchronizing the activity rhythms of these fish.

Fish in this study generally did not show an anticipatory change in activity prior to the onset of light or darkness. Byrne (1968) reported that sockeye salmon showed a "pre-dawn" increase and a "pre-dusk" decrease in activity. Pre-dark increases in activity have been observed in the nocturnal swell shark (Nelson and Johnson, 1970) and in juvenile Atlantic herring (Stickney, 1972).

Atlantic salmon showed a consistently greater response following a light to dark transition than following a dark to light change (Fig. 2). This was true even of light-active fish. Maximum activity in juvenile herring occurs at sunset at a time of critical light intensity (Stickney, 1972). Sunset is the primary synchronizer of activity rhythms of two Arctic sculpins *Cottus gobio* and *Cottus poecilopus* (Andreasson, 1969, 1973). In contrast, maximum bursts of activity occur at sunrise in brown trout, *Salmo trutta,* (Swift, 1964), the blenny, *Coryphoblennius galerita,* (Gibson, 1970) and bluefish (Olla and Studholme, 1972).

The nature of light responses in juvenile Pacific salmon is variable and complex (Hoar, Keenleyside and Goodall, 1957). Although different species exhibited preferences for either bright areas (*Onchorhynchus keta, O. gorbuscha*) or dark areas (*O. kisutch, O. nerka*), all individuals continually ventured into both light and dark regions of the test apparatus.

Juvenile Pacific salmon also change their light responses during development. Byrne (1968) found that sockeye salmon are nocturnal for the first two weeks after emergence and then become diurnal. Pink salmon fry, *O. gorbuscha,* lose their photonegative behavior after two months (Hoar, Keenleyside and Goodall, 1957). Coho salmon fry, *O. kisutch,* are indifferent to light, but the smolts are photonegative. Coho and sockeye salmon smolts are more light sensitive than the fry and seek out deeper darker areas (Hoar, Keenleyside and Goodall, 1957).

Byrne (1968) found that LL facilitated the expression of a free-running rhythm in sockeye salmon whereas DD inhibited it. This is opposite to what Ali (1964) found for Atlantic salmon. Gibson (1971) entrained rhythms in the blenny, *Blennius pholis,* that persisted in DD after several months of exposure to LD. Another blenny, *Coryphoblennius galerita,* exhibited a 12 hr tidal activity rhythm in DD (Gibson, 1970). Nelson and Johnson (1970) found that neither LL nor DD inhibited the free-running rhythm of the swell shark for about 15 days. Several fish having indications of rhythmicity in DD were noted by us and by Varanelli and McCleave (1974).

The majority of fish in some experiments was diurnal while in others it was nocturnal or light-change-active. Also, different individuals tested simultaneously in the same conditions showed different patterns of activity. Varanelli and McCleave (1974) found that slightly more Atlantic salmon tested during the summer of 1971 were nocturnal than were diurnal or light-change-active. In contrast, we found that during the following summer most fish were diurnal. During the winter and spring of 1973 the majority of fish were nocturnal or light-change-active and very few were diurnal (Table I). Because Varanelli and McCleave (1974) used various photoperiods and temperatures to approximate environmental conditions, it is possible that some interaction among photoperiod, light intensity and temperature was responsible for the difference in results between the two summers.

This does not explain why different experiments with the same conditions produced different results or why fish tested at the same time showed different behavior patterns.

Byrne (1968) found that a photoperiod-temperature interaction was responsible for changes in the activity patterns of juvenile sockeye salmon. While testing fish in several photoperiod-temperature combinations, he found that an upper temperature limit existed for each photoperiod. When this temperature was exceeded the fish changed from light-active to dark-active. High temperatures (10° C) coupled with short photoperiods (LD 8:16) caused an increase in nocturnal activity.

Seasonal changes in activity pattern have been found in Arctic populations of *Cottus gobio* and *C. poecilopus* (Andreasson, 1973), brook trout, *Salvelinus fontinalis* (Eriksson, 1972), and burbot, *Lota lota,* (Müller, 1973). The trout is day-active all year except for a desynchronized interval in summer. The sculpins and burbot are day-active in winter and night-active in summer (Andreasson, 1973). During the phase shift *C. poecilopus* passes through an interval when peak activity occurs at sunrise and sunset. Andreasson (1973) suggested that the activity rhythms of these fish are controlled by two separate oscillators, one light-active and one dark-active. A seasonal change in the phase angle between the two oscillators, caused by exogenous factors, is responsible for the change in activity pattern.

Müller (1973) found that the duration of activity in burbot in the Arctic is controlled by day length in winter and by the night length in summer. The activity time cannot exceed 10–11 hr. When the day length or night length exceeds this limit, the fish switches to nocturnal or diurnal activity, respectively.

The phase of a circadian rhythm can sometimes be shifted by a single perturbation in the light, temperature, or other regime. A single chemical stimulation via trout scent initiated a circadian activity rhythm in the adult sea lamprey which persisted in constant dim light until observations were stopped after five days (Kleerekoper, Taylor and Wilton, 1961). Fish in the present study failed to become entrained by an hour long light signal recurring periodically every 23 hr. Neither did they show the abrupt increase in activity following the light to dark transition observed in the LD experiments. Maximum outbursts of activity did not coincide with the hour of light exposure.

The hour of light exposure began in the evening and occurred an hour earlier each day over the course of the experiment (ten days). This signal may have been presented at a point in a rhythm of light sensitivity at which it could not be effective. Bruce (1960) has generalized that a light presented during an animal's subjective day may cause little or no phase shift. Because these fish have shown abrupt responses to LD transitions in other experiments, the synchronizing regime applied in this case was probably inappropriate. More information is needed about the variation in light responses and the limits of entrainability of salmonids to clarify the phase relationships between their activity rhythms and the oscillations of their environment. Indicator processes other than locomotor activity may be better suited for the demonstration of endogenous or non-visual exogenous components of circadian rhythms.

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SUMMARY

Atlantic salmon parr exposed to a 12 hr light–12 hr dark cycle (LD 12:12) for ten days were entrained to a 24.0 hr periodicity in locomotor activity. Thirty-five fish were light-active, 20 were dark-active and 32 were active primarily when lights were turned on or off. Fish maintained in constant conditions (75) were generally aperiodic. Five fish (of 30) in constant darkness (DD) showed evidence of 24.0 hr periodicity. Twelve fish exposed to a light signal of 1 hr duration recurring every 23 hr failed to become entrained. Fish in constant light (LL) showed more activity than fish in DD. The results suggest (1) that light-dark transitions are important in synchronizing locomotor activity rhythms and (2) that locomotor activity is not a good indicator of possible circadian oscillations in this species.

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DIETARY FACTORS STIMULATING OOGENESIS IN *AEDES AEGYPTI*

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Vertebrate blood provides hematophagous insects with a unique oogenic stimulus, a relationship that has long intrigued students of mosquito biology but has not been fully explained. One unresloved issue concerns the central question of the nature of the oogenic stimulus. A classical study ascribed this stimulus to the physical stretching of the midgut resulting from engorgement on vertebrate blood (Larsen and Bodenstein, 1959). However, a more recent report presents contradictory evidence and suggests that only the nutrient content of the blood-meal is crucial (Bellamy and Bracken, 1971). If this were true it would require the mosquito to assess the potential nutrient available in its midgut even before apparent digestion had begun, and this information would have to be transmitted to the brain within a few minutes of feeding (Clements, 1956). No mechanism for such a rapid assessment seems evident.

Accordingly, we re-examined this basic problem. The objective of the present study was to compare the roles of various physical and chemical properties of the blood meal in stimulating *Aedes aegypti* to commence vitellogenesis.

Materials and Methods

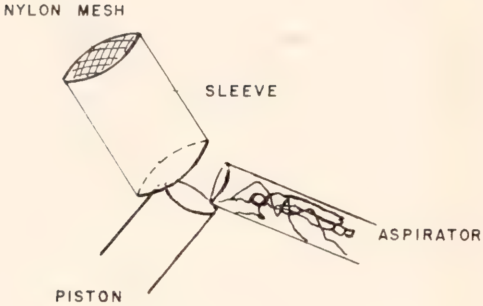
Mosquitoes were obtained from a colony of *Aedes aegypti* isolated on Grand Bahama Island in 1972 and maintained at 24–26° C, 70% R.H. and with 16 hours of light per day. Larvae were reared on Purina guinea pig chow and pharate adults separated as to sex. Virgin, female mosquitoes were provided raisins as food and used in the experiments at 3–5 days after adult ecydsis. One day prior to the experiment, food and water were removed.

In experiments requiring measurement of the quantity of blood ingested, non-anesthetized mosquitoes were transferred to a tared vial via an aspirator and weighed on a Sartorius semi-micro balance accurate to 0.01 mg. After weighing, mosquitoes were permitted to feed on a human host until suitably engorged. Immediately following feeding, each mosquito was re-weighed and transferred to individual holding chambers.

Various solutions were introduced into the mid-guts of mosquitoes in two ways: injection *via* the anus, and artificial feeding.

Injection via *the anus*

Non-anesthetized mosquitoes were transferred to an immobilization chamber by means of an aspirator (Fig. 1). The outer (sleeve) portion of the chamber consisted of a plastic tube (2 cm long and 1 cm diameter) that was closed at one end



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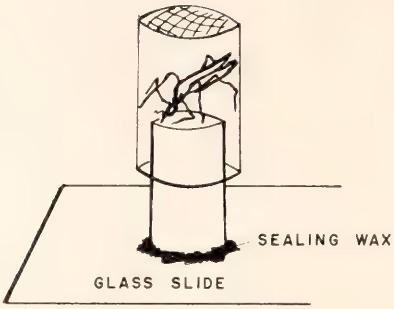

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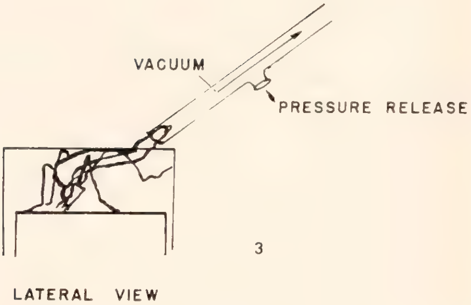

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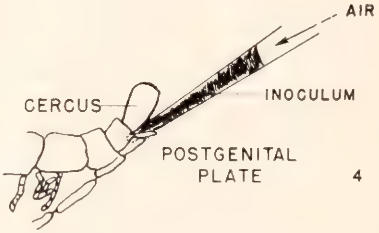

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FIGURE 1. Insertion of mosquito into sleeve portion of restraining device.

FIGURE 2. Assembly of restraining device.

FIGURE 3. Method of drawing abdomen of mosquito through mesh and extension of postgenital region.

FIGURE 4. Method for anal injection. Note that malpighian tubules are visible beneath distended pleural membrane.

by a nylon mesh (tulle). The sleeve was placed over a snug-fitting post so that a mosquito confined in the sleeve would be pressed against the mesh (Fig. 2). Suction was then applied to the mosquito in order to draw the abdomen through an opening in the mesh (Fig. 3). This resulted in eversion of the terminal abdominal segments and exposure of the anus. A finely drawn pipette was then inserted superficially into the hind gut (free-hand) and fluid expelled from the pipette by means of compressed air (Fig. 4). Resulting distension of the abdomen made it possible to visualize the malpighian tubules which became pressed against the abdominal wall. Occasionally, the gut ruptured and the hemocoele rather than the midgut, filled with inoculum. When this happened, the malpighian tubules floated freely and such mosquitoes were discarded.

Artificial feeding

Solutions were placed in 2 ml watch glasses and covered with baudrouche membrane. The watch glasses were then warmed to 37° C and mosquitoes, confined above the membrane, were permitted to feed to repletion. The ATP (0.01 M) was added as a feeding stimulant to those solutions not containing red blood cells.

Unless otherwise indicated, serum was prepared as follows: Horse serum, obtained locally, was lyophilized and re-dissolved to a desired concentration in distilled water. Resulting solutions were dialyzed for 24 hours against 0.85% NaCl (buffered to pH 7.1 with phosphate).

After feeding or injection, mosquitoes were held above water-soaked paper in individual, guaze-covered vials for two days. The ovaries of each were then removed, disrupted with a vibrating needle and examined at 430× with transmitted illumination.

The following commercial materials were employed: albumin (crystalline, bovine), albumin (5 × crystallized, egg), and adenosine triphosphate (crystalline, disodium salt) from Nutritional Biochemicals (Cleveland, Ohio); hemoglobin (2 × crystalline, bovine) and glutathion (reduced) from Sigma (St. Louis, Missouri); and globulin (human, Cohn Fraction IV) from Schwartz-Mann (Orangeburg, New York).

TABLE I

Stimulation of oogenesis in female A. aegypti *after ingestion of various amounts of human blood*

Mg. blood ingested	No. ♀♀	% with developing oocytes
0.1–0.4	16	0
0.5–0.9	49	13
1.0–1.4	42	57
1.5–1.9	21	81
2.0–3.0	17	100

RESULTS

Partial feeding

In the first experiment, pre-weighed mosquitoes were permitted to feed individually on a human host and feeding was interrupted before engorgement was complete. The weight of blood imbibed was recorded immediately upon removal from the host, and mosquitoes were sacrificed two days later. Ovaries were removed, the follicles separated, and degree of development determined microscopically. The weight of non-blood–fed mosquitoes varied between 1.9 and 3.3 mg, being influenced by the quantity of fluid in the abdomen. Weight of the bloodmeal varied between 0.1 and 4.0 mg.

Oogenesis was not initiated when mosquitoes imbibed less than 0.5 mg of blood, while all mosquitoes taking 2.0 mg or more had well-developed oocytes (Table I). Ovarian development was stimulated in about half of the mosquitoes that imbibed 1.0 to 1.5 mg of blood. Of those mosquitoes that failed to commence oogenesis, none had more than a few degenerate primary follicles, nor did secondary follicles develop when primary follicles were not stimulated.

Supplementation of partial feeding by injection

In order to determine whether distension of the midgut is prerequisite to oogenesis, saline was injected *via* the anus of partially-fed mosquitoes and ovarian development recorded after two days. Mosquitoes were permitted to feed on a human host but were removed as soon as blood could be clearly seen through the abdominal pleura. Such mosquitoes generally contained betweeen 0.5 and 1.0 mg of blood. Immediately upon removal from the host, saline (0.85% NaCl) was injected via the anus until the abdomen appeared to be fully distended. When injection was successful, blood and saline became thoroughly mixed and the total weight of blood plus saline was about 4.0 mg. During uninterrupted blood-feeding, mosquitoes normally imbibed about 2.6 mg of blood.

Of 235 partially-fed mosquitoes, 141 received saline injected *via* the anus (Table II). Almost half of these had activated primary ovarian follicles, including 13 in which most primary follicles degenerated. In contrast, of those that did not receive supplemental fluid *via* the anus, less than 12% had developing primary follicles and none of the remainder had more than a few degenerating follicles. When saline was administered *via* the anus of non-blood-fed mosquitoes, ovaries remained undeveloped.

TABLE II

Stimulation of oogenesis in female A. aegypti *after ingestion of trace amounts of blood (0.5–1.0 mg) and supplementation with saline or air injected* via *the anus*

Mg. blood ingested	Material injected *via* anus	No. ♀♀	% females with stimulated oocytes	
			Developing	Degenerating
0.5–1.0	—	112	12	0
0.5–1.0	Saline	141	38	9
0	Saline	27	0	0
0.5–1.0	Air	9	11	0
0	Air	9	0	0

Air was injected *via* the anus of other partially blood-fed-mosquitoes in order to distend the midgut without diluting blood already present there. However, this treatment appeared not to affect the developmental state of the ovary (Table II). Nor did injection of air stimulate oogenesis in non-fed mosquitoes.

Retention of injected solutions

We noted that the midgut contents of partially-blood-fed mosquitoes and of non-blood-fed mosquitoes receiving saline or air *via* the anus were generally expelled during the day following feeding or injection. This early evacuation of the gut rarely occurred following normal blood-feeding. Accordingly, we studied the relationship between serum concentration and retention of mid-gut contents. Serum was injected *via* the anus until abdomens were fully distended (about 4 mg). Unusual mortality was noted following injection of non-dialyzed, hypertonic serum.

Of 69 mosquitoes receiving twice concentrated serum, 33 died within the day following injection. In contrast, about 5% of mosquitoes died after receiving isotonic or hypotonic (diluted to twice previous volume) solutions. Accordingly, in subsequent experiments all solutions were dialyzed for 24 hours against 0.85% saline (phosphate buffered). Mortality resulting from the injection of such dialyzed, twice-concentrated solutions was about 10% while less concentrated serum produced negligible mortality. Of those mosquitoes that received serum diluted one part in ten, more than half lost the midgut contents within one day of injection (Table III). On the other hand, virtually all mosquitoes that received undiluted or twice-concentrated serum retained the inoculum.

In an attempt to prevent evacuation of the midgut, shellac was placed on the anuses of 37 saline injected and 24 air injected mosquitoes. Resulting mortality exceeded 50% during the next 2 days and the survivors were dissected at that time. Of the 12 surviving saline-injected mosquitoes, none had a distended midgut at 2 days after injection, nor were developing primary oocytes found. Instead, each mosquito had large quantities of fluid in the hemocoel and rectum. Malphigian tubules were grossly distended. Air was present in the midguts of each of the eleven surviving mosquitoes that were injected with air. It is interesting that the ovaries of six of these mosquitoes contained degenerating primary follicles. When the anus was not sealed, neither air nor saline was retained and mortality was nil; nor did the ovaries appear to be stimulated.

TABLE III

Retention of horse serum, variously diluted, during the 24 hr period after anal injection

Concentration of serum injected *via* the anus	No. ♀♀	% retaining inoculum
0.1×	35	43
0.25×	26	65
0.5×	168	89
1.0×	142	99
2.0×	133	98

Effect on ovarian development of anal injection of serum

Mosquitoes were injected *via* the anus with varying concentrations and varying volumes of serum and subsequent ovarian development noted. Volume of material introduced was determined by weighing before and after injection. Mortality remained below 5% and, since 50% was the lowest serum concentration used, virtually all mosquitoes retained the inoculum. Those few that failed to do so were discarded.

Regardless of the serum preparation used, a greater proportion of mosquitoes began oogenesis when 2.0 mg or more of solution was administered as compared to 1.0 to 1.5 mg (Table IV). No progressive increase was noted at volumes above 2.0 mg. It is interesting that ovarian follicles invariably matured (once stimulated) in mosquitoes receiving 1.0 to 1.9 mg of solution, while such follicles degenerated in

TABLE IV

Stimulation of oogenesis in female A. aegypti *injected* via *the anus with various amounts and concentrations of serum*

Mg. serum injected	Concentration of serum injected					
	0.5×		1.0×		2.0×	
	No. ♀♀	% Stimulated	No. ♀♀	% Stimulated	No. ♀♀	% Stimulated
1.0–1.9	15	33	10	30	9	33
2.0–2.9	53	51	38	66	37	76
3.0–3.9	40	55	37	73	41	85
4.0–4.9	31	52	38	63	30	77
5.0–6.0	11	55	17	59	13	77

12% of mosquitoes receiving more inoculum. No further pattern in ovarian degeneration was evident. It is clear that the proportion of mosquitoes initiating oogenesis was correlated with the concentration of serum in the inoculum (Table IV). Differential increments between twice-diluted (0.5×), normally-concentrated (1×), and twice-concentrated (2×) serum approximated 12%.

Stimulation of ovarian development by various blood components

We then administered various components of human blood *per os* and *per anus* and compared subsequent ovarian development. Those mosquitoes that did not

TABLE V

Stimulation of oogenesis in female A. aegypti *after ingestion of solutions containing various nutrients*

Solution ingested	No. ♀♀	% females with stimulated oocytes	
		Developing	Degenerating
Fresh blood	10	100	0
Stored blood	6	67	0
Blood cells	6	67	0
Serum	4	100	0
Hemoglobin-50 mg/ml	9	0	0
Albumin 10 mg/ml (Bovine)	25	4	0
50 mg/ml	36	22	0
100 mg/ml	24	38	0
200 mg/ml	12	67	0
Globulin 10 mg/ml	15	38	20
25 mg/ml	14	50	7
Glutathione 0.34 mg/ml	13	0	23
3.4 mg/ml	8	13	25
Saline	15	7	0

gorge fully were discarded. Whole defibrinated blood was highly stimulatory when ingested through a membrane although prior storage seemed to reduce this property (Table V). Similarly, oogenesis was stimulated by washed cellular components and by serum alone. ATP was added to serum preparations in order to stimulate feeding.

We attempted to identify more precisely those blood components that stimulate ovarian activity. Surprisingly, ingested hemoglobin appeared not to stimulate ovarian development (Table V); on the other hand, both bovine albumin and globulin were highly stimulatory. Glutathione appeared to be slightly stimulatory. In all but one mosquito, saline was non-stimulatory. However, in that exceptional mosquito, oogenesis proceeded as after normal blood-feeding. Degeneration of primary ovarian follicles was rarely observed in this series of observations. Degeneration occurred in few (six) mosquitoes fed globulin and a similar number (five) fed glutathione.

TABLE VI

Stimulation of oogenesis in female A. aegypti *injected* via *the anus with solutions (greater than 0.002 ml) containing various nutrients (50 mg/ml)*

Solution injected	No. ♀♀	% females with stimulated oocytes	
		Developing	Degenerating
Globulin	29	41	52
Hemoglobin	29	0	31
Albumin (egg)	18	0	0

Finally, three potentially stimulatory solutions were injected via the anus and subsequent ovarian development observed. Mosquitoes were weighed both before and immediately after injection in order to insure that each received at least 2 mg of solution. All mosquitoes in this experiment retained the inoculum at one day after treatment and more than 80% survived at two days. Globulin solutions administered via the anus stimulated oogenesis in more than half of the mosquitoes treated (Table VI). Follicles degenerated in only a few (three) mosquitoes. In contrast, hemoglobin was less stimulatory; 9 of 29 treated mosquitoes had degenerating follicles and none proceeded to mature eggs. Egg albumin was apparently non-stimulatory.

DISCUSSION

Ovarian development in anautogenous mosquitoes is arrested at a specific developmental stage, a condition normally sustained until the female gorges on vertebrate blood. Our observations on *A. aegypti* confirm previous reports (Colless and Chellapah, 1960; Roy, 1936; Volozina, 1967; Woke, Ally and Rosenberger, 1956) that resumption of development requires ingestion of a certain threshold volume of blood. A similar threshold effect has been reported for *Culex pipens* (Hosoi, 1954; Kupriyanova, 1966). It is interesting that other mosquitoes may differ in this regard; female *Culex tritaeniorhynchus*, for example, produce a few eggs when only trace amounts of blood are taken (Mogi, Wada and Omori, 1972).

These observations suggest that female *A. aegypti* may assess the quantity of blood present in the midgut by means of some volumetric measure. Since the threshold value for oogenesis appears to be about 1.5 mg of blood per mosquito and these mosquitoes normally imbibe about 2.6 mg of blood, completion of oogenesis requires that the midgut be nearly fully engorged. Indeed, when a mosquito has taken 1.5 mg of blood, it seems to be well distended and ingested blood is readily visible through the stretched pleural membrane. The oogenic signal is generated only after the mosquito is nearly completely filled.

Time relationships seem to confirm that a volumetric measure may contribute to the assessment of the nutrient content of a blood-meal. An ovary stimulating signal is generated within the head of female *A. aegypti* within 30 minutes of the completion of blood-feeding (Clements, 1956) and primary oocytes commence micro-pinocytosis and R.N.A. synthesis within an hour (Anderson and Spielman, 1971; Anderson and Spielman, 1973). At this time the mass of blood in the midgut appears to be virtually undigested indicating that an exclusively chemical-nutritional assessment is unlikely. Solely on the basis of exclusion, reception of a physical stimulus seems to be required.

Since the oogenic signal is generated after injection of nutrient through the anus, any physical estimate of food volume must involve sensations of degree of distension or of pressure within the abdomen. An estimate based on the quantity of flow through the anterior gut would be excluded. Larsen and Bodenstein (1959) have suggested that the oogenic signal is based on an assessment of the degree of distension of the midgut.

Our observations provide experimental confirmation that the volume of nutrient present in the midgut is crucial to the release of the oogenic stimulus. Eggs develop after saline supplementation of blood meals that would otherwise be too small to stimulate oogenesis. It is paradoxical that supplementation by anal injection of air fails to enhance the oogenic stimulus since, in contrast to injection of saline, injected air does not dilute the blood meal.

In addition to demonstrating the importance of physically derived oogenic stimuli, our observations confirm Bellamy and Bracken's (1971) suggestion that female mosquitoes can assess the chemical nature of their midgut contents. Working with *Culex pipiens*, these investigators found that eggs mature following repeated daily hemocoelic injection of a concentrated mixture of amino acids. Although this suggests that products of protein digestion, themselves, might transmit an oogenic signal from the midgut via the hemolymph, such non-physiologic manipulation does not constitute rigorous proof. One such digestion product, isoleucine, deserves special attention. This amino acid appears to be an essential dietary component required by *A. aegypti* for the production of eggs (Greenberg, 1951). Our observations confirm that proteins such as hemoglobulin which are poor in isoleucine generally fail to stimulate oogenesis.

Since oogenesis requires a combination of stimuli that are quite specific for vertebrate blood we might speculate that the role of the ventral diverticulum may not be to prevent stimulation of oogenesis by a sugar meal (Larsen and Bodenstein, 1959). When sugar solutions are delivered to the midgut, even in great volume, the ovaries are not affected. Indeed, unless this food happens to be isotonic with its body fluids, the insect will rapidly die. One role of the diverticulum would be to protect the midgut epithelium from osmotic stress. The wall of this inert diverticulum is highly impermeable to water (Clay and Venard, 1972) and contained fluids are only slowly released to the midgut where digestion and absorption take place.

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Summary

1. Female *Aedes aegypti* generally fail to commence oogensis unless they imbibe veterbrate blood nearly to repletion. However, oogenesis frequently proceeds if a partial blood meal is supplemented with injection of saline via the anus. Injection of saline or air alone generally does not stimulate the ovaries.

2. When the midgut is fully distended with serum the proportion of mosquitoes developing eggs is correlated with concentration of serum. Similarly, feeding on the cellular fractions of blood, on globulin and albumin fractions of serum, and to a lesser extent on glutathione, stimulates oogenesis. Hemoglobin, administered either per os or per anus is relatively non-stimulatory and egg albumin appears to be without oogenic effect.

3. These observations suggest that oogenesis depends upon distention of the midgut as well as on the presence of sufficient concentrations of specific chemical moieties.

4. A principle function of the ventral diverticulum may be to protect the midgut against osmotic stress rather than to prevent premature oogenesis.

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CURRENT-INDUCED FLOW THROUGH THE SPONGE, *HALICHONDRIA*

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The entire phylum Porifera consists of suspension-feeding organisms, simple by comparison with other multicellular animals, but nonetheless highly specialized for separating small organisms and other nutritive particulate matter from the water passing through themselves. A sponge, in Bidder's (1923, page 312) felicitous phrases, is "a mere living screen between the used half of the universe and the unused half—a moment of active metabolism between the unknown future and the exhausted past."

As Grant (1825) first showed, water passes unidirectionally through an elaborate system of pores, gates, and canals and, following filtration, is returned to the medium from which it was drawn. This flow of water through sponges has been regarded as being due entirely to the activity of certain peculiar flagellated cells, the choanocytes. Indeed, early workers such as Grant (1825) and Bowerbank (1864) devoted considerable ingenuity to demonstrations that muscles played no role.

And the general design of sponges is consistent with the requirements for a substantial output—one of Bidder's (1937, pages 129) *Leuconia aspera* Schmidt "threw, to a distance of 70 cm, 1000 times its own bulk of water per hour"—from a pump consisting of uncoordinated cells. Flagellated chambers with a combined cross-sectional area many times that of the outer surface of the animal lead into a collecting manifold in which successive generations of channels are both fewer in numbers and of smaller total cross-section. Thus the velocity of the final excurrent stream through the osculum may be several hundred times that at the level of the choanocytes. The reduction in cross-section of the final common canal at the osculum has been interpreted as a further device to increase the excurrent velocity, and the oscular chimneys have been regarded as a means of increasing the distance between excurrent and incurrent openings. Both features presumably reduce the likelihood of recycling previously filtered water (Bidder, 1923; Leigh, 1971).

But this generally accepted picture may, in fact, overlook another important element propelling water through at least some sponges under certain circumstances. Previous observations of the pumping activities of sponges were made with the animals in still water (except for the field work of Reiswig, 1971). Yet sponges appear to require moving water in their normal habitats; DeLaubenfels (1954) feels that velocities of two or three kilometers per hour are optimal. This requirement has been interpreted as insuring that silt and debris do not accumulate and as further insurance against reingestion of a sponge's output. But, whatever its other functions, *the presence of a current in the medium around an attached sponge raises the possibility of flow through the animal without active pumping by the choanocytes.* Such induced flows have been termed "passive" since they do not entail immediate metabolic cost (Vogel and Bretz, 1972). The present investigation seeks to determine whether passive flow might be an appreciable factor in the lives of sponges.

It should be noted that passive flow, while it may avoid metabolic expenditure, involves no novel source of energy. A potential exists between any two points where the velocity of the medium is different; where a potential exists, work may be extracted by an appropriate device. For an attached organism, the velocity at the point of attachment is, of course, zero; and it can extract energy from the flow of fluid around it just as does a windmill.

At least three physical mechanisms permit the induction of flow in a spongelike structure by motion of the external medium (Fig. 1). (A) If a small pipe connects two points in a larger channel, and if the ends of the small pipe are normal to the walls of the channel, then fluid will flow in the small pipe from the end where flow in the channel is slower to the end where flow is more rapid (1a). By Bernoulli's principle, the increase in velocity in the channel is concomitant with a reduction in pressure in order that energy be conserved; this pressure difference, then, induces flow in the small pipe. The direction of flow in the channel is, of course, inconsequential. With sponges, the medium should travel fastest as it crosses the highest points, which commonly bear the oscula. Thus the internal flow will be directed from any ostia not atop a major protrusion toward any terminal oscula on the protrusions.

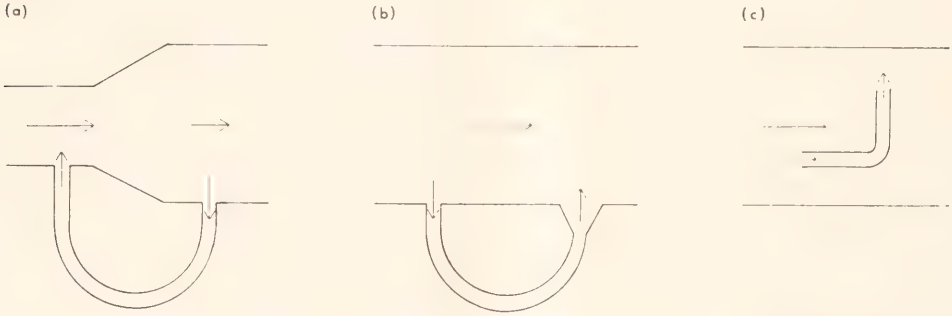


FIGURE 1. Several arrangements whereby flow in a larger channel may induce unidirectional fluid movement in a small pipe.

(B) If a small pipe terminates normal to the wall of a larger channel through which fluid is moving, then fluid will be drawn from the pipe into the channel by an additional agency, termed viscous entrainment or sucking, and caused by the resistance of real fluids to rapid shear rates. Faster movement of the fluid in the channel will produce greater rates of entrainment (1a). In addition, the rate of entrainment will depend on the size of the aperture (1b); it is to avoid errors due to entrainment that the static (normal) aperture on pitot tubes (as used for airspeed indication in small planes) must be kept small (Prandtl and Tietjens, 1934). In a finger-like or encrusting sponge, water will therefore be drawn out of a terminal osculum because, as the highest point on the structure the latter will be exposed to the greatest velocity, as well as because the oscula are larger holes than the ostia.

(C) If a small pipe bent at a 90° angle is oriented in a moving stream so that one aperture is directed upstream and the other is normal to the stream, then fluid will enter the former and run out of the latter (1c). The normal aperture "sees" only the static pressure of the fluid; the one directed upstream is exposed to the sum of the static pressure and the additional dynamic pressure caused by the deceleration of the fluid in front of the aperture. In a finger-like sponge, an ostium directed upstream will thus be exposed to a higher pressure than will an osculum normal to the flow. If the external flow reverses direction, then ostia on the opposite side of the finger will be exposed to the higher pressure, and internal flow will still be from ostia to oscula.

For a sponge with a terminal osculum, all three mechanisms predict the same direction of flow—from ostia to oscula. It has not yet proven feasible, either by theory or measurement, to apportion the passive flow among the different mechanisms which might be responsible. One can, however, state that size, shape, and location of the openings should all be relevant to determining the direction of passive flow. Consideration of these possible physical mechanisms suggests that a good excurrent opening should be large, at the terminus of a fairly sharp projection, and farthest from the point of attachment to the substratum.

That a saving in the energy expended in filtering water might be important to sponges is evident in the calculations of Jorgensen (1966). His figures, based on filtration rates and oxygen consumption in still water, suggest that the nutritive value of the suspended material available may not greatly exceed the cost of filtration.

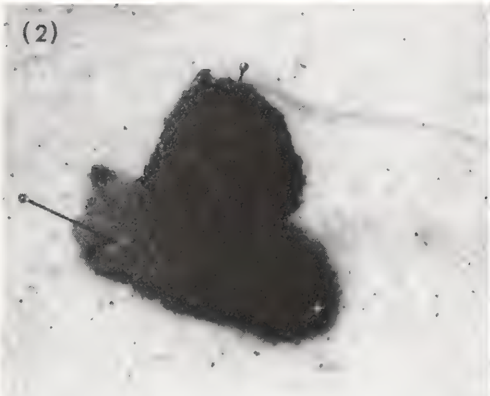
Passive flow makes a significant contribution to the ventilation of the burrows of prairie-dogs (Vogel, Ellington and Kilgore, 1973) and mounds of certain African termites (Weir, 1973), and it may have been crucial to the success of archeocyathids in the Cambrian (Balsam and Vogel, 1973).

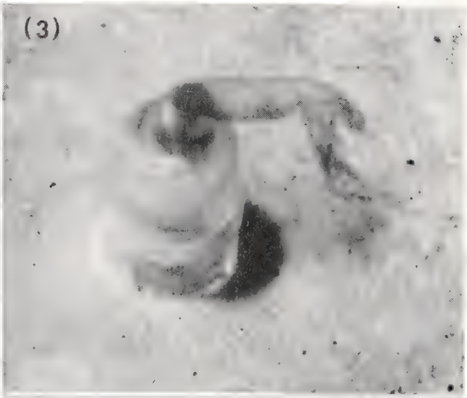
Ideally, an evaluation of passive flow in sponges should be based on direct observations in nature and on a theoretical analysis of the hydrodynamics of the situation. Direct observation, however, has given ambiguous results due to the complexity and variability of the currents around the sponges investigated. And theoretical analysis has been frustrated by the complexity of the physical mechanisms involved. An operational compromise, the present investigation divides into three experimental portions: first, observation and measurements of flow through living and freshly killed sponges exposed to moving sea-water in the laboratory; secondly, measurements of flow rates through a brass model of a sponge in a flow tank; and thirdly, observations on the influence of the geometry of apertures on the induction of flow.

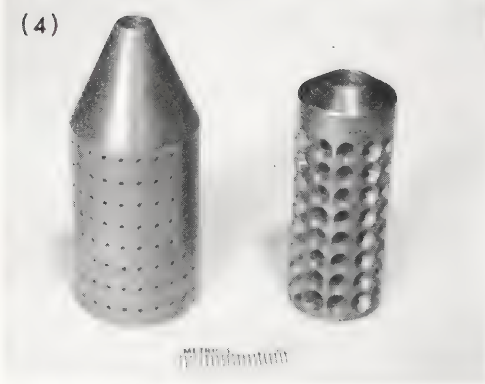
MATERIALS AND METHODS

Fresh material

Sponges (*Halichondria bowerbanki* Burton: see Hartman, 1958) were collected by the Supply Department of the Marine Biological Laboratory, the encrusting form near Woods Hole, Massachusetts, the more erect and finger-like







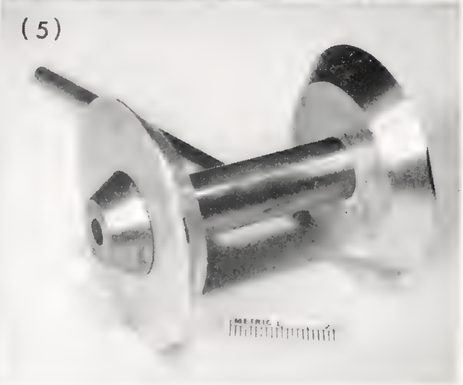


FIGURE 2. Rhodamine B passing through a freshly killed *Halichondria* at an external current of about 5 cm/sec.

FIGURE 3. Rhodamine B passing through a plexiglas model (described by Vogel and Bretz, 1972) under the same conditions as in Figure 1. The model is 2.5 cm high.

FIGURE 4. Brass model on which the data of Figure 8 were obtained. The smaller cylinder inserts into the larger through a hole in the bottom of the latter. Two of the small holes in the outer cylinder communicate with each chamber in the walls of the inner cylinder; thus holes in outer and inner cylinders are not aligned.

FIGURE 5. Apparatus for testing apertures in pairs. Each aperture is radially symmetrical and was oriented normal to the stream; dye was introduced through the small pipe, which also served as a support. Large collars on either end of the main tube reduced the sensitivity of flow through the tube to minor misalignments in the stream.

form from the Cape Cod Canal. All were maintained in running sea water and used within a day of collection. In the erect form, active pumping usually ceased within a few hours after the sponges were received, probably due to the warmer water at the laboratory.

With the addition of curved corner fairings, a sea-table, 132 × 76 × 10 cm inside, provided an adequate flow tank for use with the fresh sponges. The supply hose, fixed to one wall and directed downstream, propelled water at up to 10 cm/sec. Water left through a porous cylinder fixed to the drain, thereby providing a continuously changing medium in the tank and facilitating observations of the patterns of flow with dye markers. Of several colored materials used, a concentrated solution of rhodamine B in sea water was by far the most suitable for marking flows; in practice it was introduced from a hypodermic syringe, and it stained neither plastic models nor sponges.

"Fingers" of *Halichondria* were cut from colonies with a substantial portion of the basal portion of the colony left attached and without cutting into the axial spongocoel; these were pinned to wax in a small finger bowl. Above the wax, a small amount of sand provided convenient fairing material to obscure the cut end of the sponge and to raise the substratum to the level of the rim of the bowl (Fig. 2). After introduction into the sea table, each specimen was checked with a dye marker for active pumping; several either stopped pumping shortly thereafter or never showed evidence of pumping. Certain of the latter were used for measurements of passive flow. Active pumping, when occurring, was steady, with none of the polyrhythmic character of the ventilatory flow of, for example, burrowing polychaetes. No specimen which spontaneously stopped pumping was ever observed to start again. For most measurements of passive flow, sponges were deliberately "turned off" by immersing them in fresh water for about five minutes. Parker (1910) found that this treatment eliminated active pumping without causing constriction of either ostia or oscula in *Stylotella*. Fresh water produced no visible effects on the openings of *Halichondria*, and the performance of sponges which had spontaneously stopped pumping was indistinguishable from those treated with fresh water.

For measurements of flow inside a specimen, a probe (see below) was lowered about 4 mm below the tip of the osculum; the same probe was repositioned about 3 cm in front of the sponge to record the outside velocity. Traverses from the level of the osculum down to the sand fairing showed an essentially constant velocity—the use of a finger-bowl to hold specimens reduced the boundary-layer thickness to insignificance.

Models

Models were made from cylindrical metal or plexiglas stock on a combination lathe and milling machine.

A linear flow tank filled with fresh water was employed for measurements on all models. This device consisted of a plywood channel, 244 × 23 × 17 cm, connecting two 200-liter, open cylindrical drums. A 1.5 HP centrifugal pump in the return circuit provided flow rates of about 7 l/sec or (depending on the depth of water in the channel) up to 40 cm/sec. An adjustable bypass around the pump together with an adjustable gate at the downstream end of the channel controlled the flow rate and depth in the channel. Flow was smoothed by baffling in the fore-tank and by an array of small pipes acting as a straightener at the entrance to the channel. These devices proved adequate: a blob of dye remained visually discrete as it travelled the length of the channel. More water passed through the lower portion of the adjustable gate than through the upper portion, thus compensating for the retardation caused by the floor of the channel. As a result, the speed of flow was nearly uniform from within a few millimeters beneath the surface to about a centimeter above the bottom at the place, about half-way down the channel, at which all measurements were made.

Flow-meter

Flow rates were measured with a heated-bead thermistor flow-meter (Figure 6) whose accuracy was estimated (on the basis of repeated calibrations) as ±0.5 cm/sec between 0 and 30 cm/sec. Several limitations on the use of this instrument deserve comment. In the absence of a temperature-compensating circuit, measurements had to be made at water temperatures differing no more than 1° C from that of the calibration. The instrument had a substantially non-linear response, giving a greater change in output for a given change in flow rate at low velocities. The thermistor bead together with its glass envelope on the end of the probe was 2.0 mm in diameter while the diameter of the cylindrical shaft of the probe was 1.5 mm; thus the probe seriously occluded oscula less than about 3 mm across.

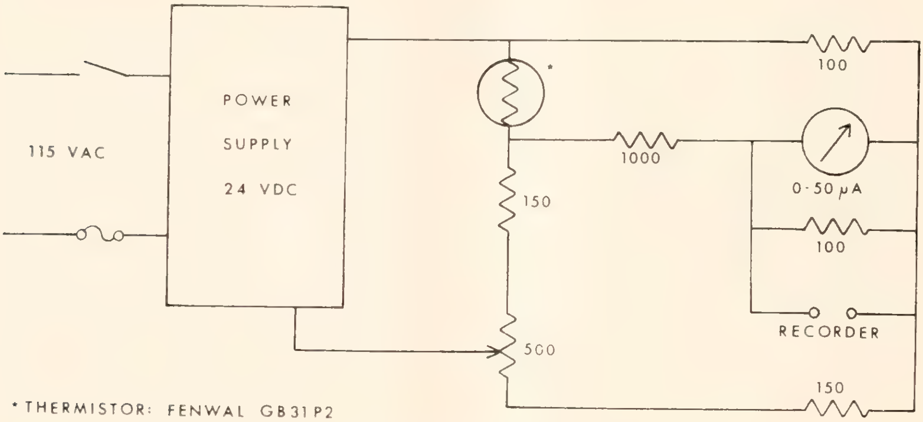


FIGURE 6. Circuit diagram of the thermistor flow-meter used for measurements in liquids at low speeds.

Finally, the probe could not be used in confined spaces (such as a spongocoel or oscular channel) at speeds below one cm/sec without inaccuracy resulting from local heating of the medium. The flow-meter was, however, not appreciably directional in its response; and its response time of about one second was amply short for present purposes.

For measurements on fresh material, the flow-meter was calibrated with the probe in a motor-driven revolving cylindrical bowl of sea water, 20 cm in diameter and 10 cm deep. In practice, the bowl was rotated until the water inside was moving at the same angular rate as the bowl itself; then the probe was lowered into the water near the center and slowly moved radially outward while the output of the meter was recorded. If the probe remained in one position for more than a few seconds, local heating and/or retardation of the medium gave a spurious reduction in the output of the meter.

For measurements on models in fresh water, the flow-meter was calibrated in the linear flow tank simply by measuring with a stop watch the time necessary for a dye-marker to travel 150 cm and comparing the result with the output of the meter.

Results

Fresh material

Most of the actively pumping sponges gradually reduced their activity during the course of the measurements in the flow tank; therefore it was necessary to determine the pumping rate in still water after every few measurements of total flow. Data from animals whose rates declined were discarded on suspicions of moribundity. Figure 7 shows the combined active and passive flow for a sponge whose pumping rate in still water did not vary detectably over the course of the measurements. It continued this rate of pumping for an hour afterwards, suggesting that the data for total flow through the sponge may be representative of flow rates in the normal habitat. Other specimens gave similar, but less regular and reproducible results.

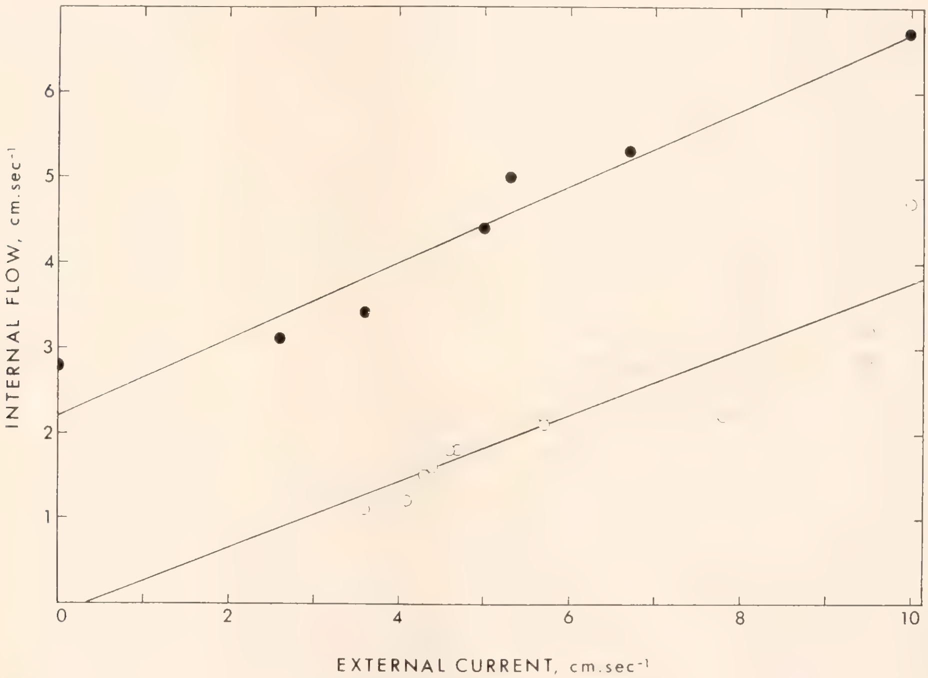


FIGURE 7. Flow across and through specimens of *Halichondria*. Closed circles—active pumping; open circles—specimens "turned off" by exposure to fresh water. The regression equation for the upper line is $Y = 0.450 \times +2.207$; for the lower line it is $Y = 0.390 \times -0.124$.

Figure 7 also shows flow rates for sponges which, as determined by observations with dye markers, were not actively pumping. Although always less than the total flow through active sponges, these rates were substantial: at an external current of 7 cm/sec, the passive component was typically about one-half the total flow through an actively pumping sponge.

Neither the x nor y intercepts of the line fitted to the data on passive flow differ significantly from zero: at least in this range of speeds there is no evidence of either residual pumping or of a non-linear relationship between internal and external flows. Moreover, the slopes of the lines for total flow and for passive flow through the sponges do not differ significantly: the active component appears to be simply "added on" to the passive flow and its magnitude does not depend on the strength of the external current in this range of speeds.

For the sponges for which data are presented here, mean internal oscular diameter was 4.9 mm (S.D. = 1.5, n = 7). The individual fingers were 31.0 mm (S.D. = 4.7) high and 12.1 mm (S.D. = 3.5) in outside diameter half-way between base and tip.

A plot of external current *versus* passive flow through a sponge gives a convenient measure of the resistance of the animal to the induction of flow through it. The reciprocal of the slope provides a dimensionless "resistance index" which lumps all of the factors affecting the efficacy of passive flow; for these sponges, the resistance index is 2.56. Comparisons with other species and specimens from other habitats should be of interest.

Observations on the encrusting or "hard" variety of *Halichondria* were made with dye markers, since the oscula were too small to permit insertion of the thermistor probe. Qualitatively, results differed in no way from those obtained with erect, finger-like specimens. Both active and passive flow were clearly evident, the former in still water and the latter after the usual exposure to fresh water to inactivate the choanocytes.

Model sponges

The results obtained on fresh material, actively pumping and "turned off" imply that the so-called passive component of the flow through a sponge is a purely physical phenomenon, independent of movement of any part of the animal. Further evidence that active processes need not be invoked emerges from observations and measurements on completely non-living, physical models of sponges.

A visual comparison of a "turned off" fresh sponge and a plastic model is provided by Figures 2 and 3. In both cases, a solution of Rhodamine B was injected near the upstream side of the basal end of sponge or model a few seconds before the picture was taken. The similarity of the pattern in which the dye solution emerges from the oscula is clearly evident.

A quantitative view of the flow induced in a model is provided by Figure 8. This model (Fig. 4) incorporates analogs of incurrent and excurrent canals as well as flagellated chambers, although it is still a very crude imitation of the array of channels in even a simple sponge. In particular, it proves impractical to reproduce the minute dimensions and enormous numbers of internal channels of a sponge, and it is convenient to construct a model somewhat larger than the size of the *Halichondria* fingers. This model is about twice the height of a real finger, and thus an equivalent flow is achieved when the external current passing the model is half that passing a real sponge.

The behavior of model and real (turned off) sponges are, nevertheless, quite similar; even the resistance indices (2.30 and 2.56) do not differ much if the data obtained on the model with external currents below 11 cm/sec are considered. It appears that the larger size of the channels in the model roughly compensates for their fewer numbers.

The principal difference is the lower slope of the curve for the model obtained at external currents above 11 cm/sec. Limitations of the sea-water flow tank precluded making equivalent measurements on fresh sponges, particularly since the appropriate velocities would have had to be above, not 11, but 22 cm/sec. However, the reduced slope is most likely to be an artifact of this particular model. When a very loosely-fitting and porous collar shielded the cylindrical portion of the model from an external current above 11 cm/sec, the flow through the model increased. At lower external currents, the collar was without effect. Perhaps the unrealistically large diameters of the "ostia" in the model permitted significant entrainment at the higher speeds, thus opposing the entrainment at the osculum.

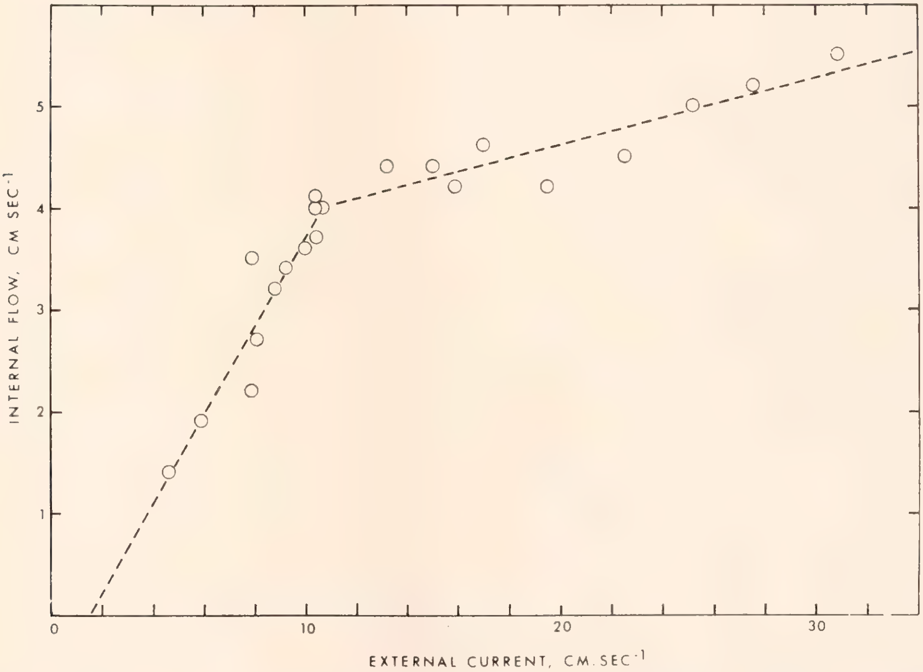


FIGURE 8. Flow across and through the model shown in Figure 4. Below an external current of 11 cm/sec the regression equation is Y = 0.436 × −0.647; above 11 cm/sec it is Y = 0.066 × +3.29. Flow was undetectable (less than one cm/sec) through the model when the "ostia" were blocked.

In addition to the model sponges shown in Figure 3 and 4, several others were constructed and tested with dye markers for passive flow, the latter by members of the Experimental Invertebrate Zoology Course (1972) at the Marine Biological Laboratory. Ms. Sarah E. Swank made a conical, crater-like model, 3 cm high, with a ring of large collecting channels directed upward and entering the common spongocoel just below the osculum. Mr. Duen Yen devised a vase-like model, 12 cm high, consisting of a wire framework wrapped with percale cloth, and producing more numerous and tinier pores than could be made of metal. Both of these models permitted substantial induction of internal flow by movement of the medium. Indeed, our impression, based on a total of six "sponge" and two "archeocyathid" (Balsam and Vogel, 1973) models, is that effective designs may vary greatly in their details. The basic constraints of the scheme are little more than small input pores and a large output opening, the latter, at least, exposed to a transverse current. It may prove to be the case that sponges of a wide variety of shapes and sizes eventually show evidence of significant passive flow.

Model oscula

The oscula of sponges are not limited to sharp-edged, chimney-like holes at the ends of fingers, as in these *Halichondria.* Rather, they display considerable diversity, from rimless openings on the flat surfaces of some sponges, to small, crater-like elevations distributed over a branching array of cylinders, or holes on the inner surface of large, basket-shaped sponges (see, for example, DeLaubenfels, 1950). Measurements on models of the mounds of prairie-dogs (Vogel, *et al.,* 1973) showed that the geometry of the openings is important in determining the magnitude of induced flow. The shape of the exit hole is of particular consequence, apparently in connection with the phenomenon of viscous entrainment.

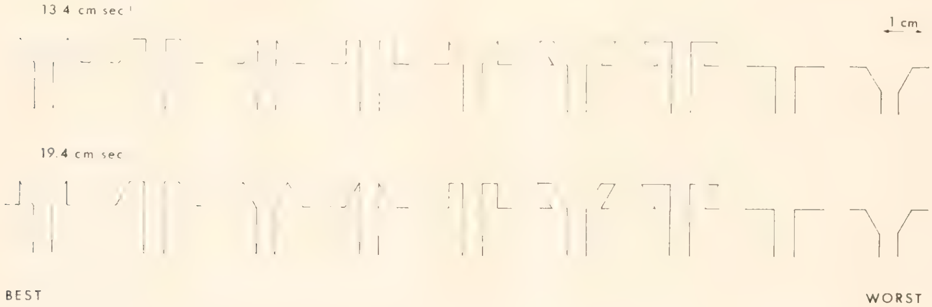


FIGURE 9. Ordering of test apertures from most to least effective exits at two different currents; results obtained with the apparatus of Figure 5.

Nine apertures were arranged from best to worst exits by testing one against another in enough combinations to produce an unambiguous ordering. Figure 5 shows the apparatus used for these comparisons. The results, given in Figure 9, prove rather complex. In general, sharp-edged openings are better exits than are blunt openings, and apertures which protrude from the surface are better than openings flush with the surface; this is essentially the same picture which emerged from the investigation of prairie-dog mounds. But the details of choices among internal tapers, external tapers, or combinations of the two prove dependent on, at least, the velocity of the external current. The dependence on velocity certainly indicates a relationship with the Reynolds number (size of object times density of a medium times velocity divided by viscosity). Thus it appears that a wide variety of shapes may be effective in inducing flow through a sponge, and that the optimum exit for a particular circumstance should depend in a complex manner on the overall shape and size of the sponge and on the currents in its habitat.

Discussion

A simple maneuver, immersion in fresh water, permitted observations of induced flow in the absence of choanocytic pumping. But all sponges, as far as is known, do have choanocytes; and, it appears, they pump water with only occasional periods of quiescence (Reiswig, 1971). How, then, are passive flow and the operation of the choanocytes interactive?

According to the present data, the induced, passive flow achieves a magnitude comparable to that of the active, pumped flow at very low rates of movement of the surrounding medium. The induced rate matches the active pumping rate of about 2.5 cm/sec when the medium is moving at slightly under 6 cm/sec; above this value, the passive component of flow through the sponge exceeds the active rate. The active rates reported by others are somewhat greater than that found here: Parker (1914), Bidder (1923), and Reiswig (1971) mention rates most of which are between 5 and 15 cm/sec. Still, the rate at which fluid passes the choanocytes, even when they are fully active, should be strongly dependent on the rate of movement of the medium.

Furthermore, the difference between passive flow and total flow through the sponge is substantially independent of the rate of flow of the medium; in short, the active component appears to remain constant. No evidence was found suggesting that the choanocytes reduce or stop their flagellar activity at high rates of flow of the medium, and none was expected following Reiswig's (1971) observation that the quiescent periods do not correspond to unusually high (or, for that matter, low) currents in the surrounding sea. The constancy of this difference between passive flow and total flow suggests that the efficacy of the choanocytes as pumps is not dependent on the rate of flow past them.

By analogy with simple mechanical pumps, one might expect that, as the flow rate through the sponge increases, the contribution of the choanocytes would be reduced in absolute value as well as a fraction of total flow. Finding the opposite result raises the suspicion that the flagella operate in a manner analogous to a variable pitch propeller: perhaps the rate at which waves propagate along a flagellum is a function of the local flow rate. Similarly, one might expect that it would be energetically advantageous for a sponge to deactivate its flagella at high rates of passive flow. But, of course, sponges are preeminently filterers, and perhaps the filtering function of the choanocytes requires continued flagellar action.

As mentioned earlier, passive flow in sponges can result from the operation of several physical mechanisms, either singly or in combination. While the present data do not permit us to distinguish the particular contribution of each mechanism, Bidder's (1923) elaborate measurements on a specimen of *Leuconia* (referred to in the paper as *Leucandra*) permit some suggestive calculations. Application of Poiseuille's law,

$$Q = \frac{\pi \Delta P a^4}{8\ \mu l} \tag{1}$$

(where Q is total flow, a is the radius and l the length of a pipe, and μ is the viscosity of the medium) to his data yields a pressure drop of 67.2 dynes/cm^2 for his observed flow rate of 8.5 cm/sec at the osculum in otherwise still water. The first and third mechanisms for passive flow described in the Introduction depend on the operation of Bernoulli's principle. According to the latter

$$\Delta P = \tfrac{1}{2}\rho V^2 \qquad (2)$$

(where ρ is the density and V the velocity of the free stream). If we assume that one opening or set of openings (here, the ostia) are at stagnation points or otherwise still water of the same total head of pressure as the free stream and further assume that the other opening (here, the osculum) is normal and exposed to the free stream, then a velocity of 11.6 cm/sec across the osculum would produce the requisite pressure drop of 67.2 dynes/cm^2.

Bidder's *Leuconia* and the present *Halichondria* are not dissimilar in structure; the former are merely somewhat longer (10 cm) and narrower (0.8 cm) than the latter (3.1 × 1.2 cm). Moreover the velocities (8.5 cm/sec through the sponge and 11.6 cm/sec across it) are not substantially different from the present results. Thus the assumptions that pressure is generated according to Bernoulli's principle and resisted according to Poiseuille's law might account for the magnitude of the passive flow observed.

But these assumptions do not explain the influence of oscular geometry on passive flow; the latter seems more in accord with an important role for viscous entrainment (the second mechanism in the Introduction). Moreover, if the expression for pressure drop (2) is substitued for the pressure in (1), it becomes clear that flow through the sponge should be proportional to the square of the velocity of the medium. In fact, the relationship (Fig. 7) proved closer to the first than to the second power. A mechanism based on the viscosity of the fluid, such as entrainment, could account for the greater than expected efficacy of passive flow at low speeds. Still, the exact dependence of passive upon external flow is not decisive, since any flow through the sponge will relieve the pressure drop calculated by Bernoulli's equation, greatly complicating the analysis.

If flow can be induced in a sponge with the modest external currents used here, the more rapidly moving water of most sponge-occupied habitats (see DeLaubenfels, 1954) should cause substantial flow. Yet the phenomenon of passive flow has hitherto escaped attention. Has any previous investigator made observations which should have disclosed a passive component of flow through sponges? Perusal of the relevant literature has turned up no case in which appropriate observations failed to detect passive flow. The usual difficulty seems to have been that work on flow through sponges has been done in a laboratory in otherwise still water.

Reiswig (1971), however, carried out extensive field study of the pumping activity of tropical Demospongiae; this latter work thus merits close attention. Both short and long term records of pumping were made, with continuous recording of oscular velocities for up to six days and sequential measurements on some specimens for up to three month periods. Unfortunately, his current recorder had a response time of about 30 seconds, so changes in flow through a sponge as individual waves passed were not detectable. Also, he reports no measurements of ambient currents. Reiswig does report that, for his main shallow-water species, "maximum pumping activity occurs when water circulation over the habitat is maximal."

Furthermore he mentions that thick-walled sponges with high pumping velocities are common in high turbulence areas on the outer reefs, while low-velocity, thin-walled forms predominate in low-turbulence regions. If turbulence is equated with ambient current strength, this distributional pattern could constitute evidence of adaptation to the use of passive flow: where the water moves more rapidly across a sponge it could induce flow through a greater thickness of wall.

To make use of induced flow, a sponge must have its osculum exposed to moving water. To penetrate the boundary layer of semi-stagnant water and expose its osculum, a sponge will have to protrude further from its surface of attachment if the ambient movement is slight than if the current is swift. It is therefore significant that from Grant (1825) onward, observers have noted the expected relationship (both inter- and intraspecifically) between the shapes of sponges and the characteristics of their habitats. For example, concerning *Halichondria panicea*, DeLaubenfels (1949, page 17) states, "Oscules conspicuous, 1–4 mm in diameter. The tissues of the sponge are elevated around these vents, so that they resemble craters of tall volcanoes. There is a rough correlation between the speed of currents at the sponge's location and the height of these volcanoes. Where the surf dashes back and forth, *panicea* is nearly smooth, but where it grows in comparative calm, the oscular chimneys may be elevated two or even five cm. In the latter case the width at the base of the tower is about 1 cm." The same author (1947, page 39) comments more generally, "Currents certainly modify sponge shape. One may notice where rocks are pounded by surf, that the sponges are regularly of the encrusting habitus. Elsewhere some of the same species may have the same shape, and give indication that such is the inherent tendency of the species, but many kinds of sponges, when in calmer waters, reveal their inherent tendency to proliferate and to have a ramose or otherwise erect shape." Such statements, although not definitive, clearly suggest that passive flow is of some importance in nature.

The principal direct evidence of passive flow in the normal habitat of sponges is the qualitative observations of M. C. LaBarbera and D. I. Rubenstein, Duke University, Department of Zoology (personal communication) in the Florida Keys. They watched a solution of methylene blue be taken up from around basket and columnar sponges with terminal oscula alternately exposed to strong surges and relatively calm water in a six to ten second cycle. The flow rate through the sponges was markedly dependent on the ambient current, being very low during the calms and high during the surges. They also noted that any columnar sponge with several vertical columns was invariably oriented with the columns in a row perpendicular to the direction of the surge; the latter arrangement should maximize exposure to moving water.

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SUMMARY

1. If a sponge is exposed to moving water, it is physically possible for water to pass unidirectionally through it without active pumping by the choanocytes; mechanisms presented for such "passive flow" predict flow from ostia to oscula.

2. In actively pumping *Halichondria,* water passes more rapidly from ostia to oscula in the presence of ambient currents; water flows through freshly killed *Halichondria* only when the surrounding medium is in motion.

3. Quantitatively similar passive flow may be induced by motion of the medium in a variety of physical models of sponges.

4. The efficacy of the induction of passive flow depends in a complex manner on oscular geometry although, in general, sharp-edged, protruding apertures are the most effective exits.

5. These apparently novel results are consistent with much earlier literature on the structure, behavior, and ecology of sponges.

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THE VERTICAL DISTRIBUTION AND REPRODUCTIVE BIOLOGY OF *PELOGOBIA LONGICIRRATA* (ANNELIDA) IN THE CENTRAL ARCTIC OCEAN

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Although holopelagic polychaetes are widely distributed throughout the oceans, very little quantitative information exists on their vertical distribution and even less is known of their reproductive biology and life histories. Most of our knowledge on these subjects comes from the many expeditions and surveys of the last one hundred years which, although of great importance in many respects, are of limited value for analytical evaluations and comparisons. This is due to differences in the type of collecting gear and sampling methods employed and in the depths sampled, and the fact that most cruises covered too wide a geographic range to intensively study any one area over an extended period of time. As a result the information which can be obtained on any particular species from a review of such data is necessarily incomplete and sometimes even contradictory. More recent quantitative approaches to plankton analysis have not increased our knowledge either, since they have generally not included pelagic polychaetes. Of the few studies on holopelagic polychaetes in which closing nets have extensively been used, Mileikovsky (1969 and 1962), Tebble (1960), Friedrich (1950), and Hardy and Gunther (1935) deserve special mention. Of the polychaetes studied by them, one of the most abundant was *Pelogobia longicirrata* Greeff 1879, a cosmopolitan species which reaches a maximum length of approximately 12mm in 26 to 30 segments and has been collected from below 4000m to the surface and throughout the world: Støp-Bowitz, 1948 (north Atlantic); Friedrich, 1950 (Atlantic); Tebble, 1960 (south Atlantic); Hardy and Gunther, 1935 (South Georgia); Augener, 1929 (Weddell sea); Berkeley and Berkeley, 1964 (Peru); Dales, 1957 (central Pacific); Tebble, 1962 (north Pacific); Mileikovsky, 1969 (northwest Pacific); and Okuda, 1937 (Japan). For a more complete listing of the literature concerning this species the reader should see Dales (1972).

P. longicirrata is also present in the central Arctic ocean (Uschakov, 1957; Knox, 1959; and Yingst, 1972) and was the most abundant holopelagic polychaete collected in a two year program of systematic plankton sampling conducted from Fletcher's Ice Island T-3. This paper presents data from that two year study on the reproductive biology and vertical distribution of the larvae, juveniles, and adults of *P. longicirrata* and proposes a life history for this species. Fletcher's Ice Island T-3 served as a stable working platform which permitted long term quantitative sampling with closing nets in a restricted oceanic region and thereby allowed researchers to amass the type of data so difficult to obtain by more conventional means in the open sea. Likewise, the direct development of the larvae of *P. longicirrata* into a form clearly recognizable and comparable with the adults (Nolte, 1938) has facilitated this study. Since *P. longicirrata* is an abundant cosmopolitan species, it is hoped that this Arctic study will also be of general interest to those concerned with the distribution and natural history of holopelagic polychaetes in other seas.

Materials and Methods

A one meter closing net with a number 6 mesh (215 μ opening) was used to sample discrete vertical intervals from 0 to 1500 m in the central Arctic basin. All sampling was done by oceanographic teams from the University of Southern California and the University of Washington from Fletcher's Ice Island T-3, while the island drifted from 84° 24′N, 112° 30′W (March, 1970) to 84° 20′N, 86° 17′W (December, 1971). Complete series of tows from 0 to 1500 m were taken an average of 2 or 3 times a month from March, 1970 to November, 1971 with the exception of September, October and November, 1970. During 1970 the upper 300 m (0–300 m) was sampled in 50 m increments; successive tows of 200 m were taken from 300 m to 900 m followed by 300 m tows from 900 m to 1500 m. In 1971 the sampling program from 0 to 500 m was modified, such that 25 m tows were taken from 0 to 400 m, and 100 m tows from 400 m to 500 m. For purposes of this report, data in the upper 500 m from both years was pooled to give three intervals: 0–100 m, 100–300 m, 300–500 m. Thus, a complete series of tows from 0 to 1500 m includes the following: 0–100 m, 100–300 m, 300–500 m, 500–700 m, 700–900 m, 900–1200 m, 1200–1500 m. All data presented here are from complete series of tows with the exception of July/August, 1970 when there were no tows from 1200–1500 m and December, 1970 when the results from one complete series from 0–1500 m were extrapolated to a partial second series extending from 0 to 700 m. In this case, the same number of specimens found in each size class and in each depth interval from 700 m to 1500 m in the complete series was added to the data from the partial series from 0 to 700 m. The net result is the adjusted equivalent of two complete series for December, 1970.

Each tow was fixed and preserved in 10% formalin in seawater. Later, the USC Arctic project personnel sorted the animals to phylum and gave the annelids to the author who identified them to species. The total number of complete specimens and fragments bearing a prostomium of *P. longicirrata* were then counted and separately recorded for each tow. Likewise, the number of true segments (the entire worm excluding the prostomium and the pygidium) was determined for each complete specimen in a given tow. On the basis of its number of true segments, each animal was placed in one of 8 size classes. Each class contained specimens in a three segment range, so that the first group included specimens with 1 to 3 segments, the second those with 4 to 6 segments, and the eighth those with 22 to 24 segments.

In order to compare the vertical distribution of the size classes throughout the year, the above data for each vertical tow were grouped into the depth intervals previously noted and pooled for a two month period. Due to the differences in the lengths of the depth intervals comprising the series of tows from 0–1500 m, the volume of water filtered by the net varied considerably from interval to interval. To remove this volume bias in comparing the data in the different intervals, the number of animals in each size class in the 100 m intervals was multiplied by 3 and those in the 200 m intervals by 1.5, so that the volume of water equalled the amount filtered in the largest intervals of 300 m. After this correction, the number of animals in each size class at each depth was determined as a percentage of the total number of complete specimens in a given two month period.

The above manipulation of the volume filtered was possible because the filtering efficiency of the net was not found to vary considerably between tows. The filtering efficiency was calculated by dividing the amount of water actually filtered by the net (as measured by a flow meter) by the maximum theoretical filtering capacity of the net. The ratio averaged approximately 0.5.

The average density of animals from 0–1500 m was calculated by dividing the total number of complete and incomplete specimens in a two month period by the total volume of water filtered by the net, as calculated from the filtering efficiency and the length of the tow.

Oocytes were counted through the body wall under a compound microscope and measured with an ocular micrometer. If an animal had a minimum of 4 oocytes in each of at least 3 segments, it was recorded as being ovigerous. The total number of ovigerous animals taken in a given two month period was then figured as a percentage of all the specimens with more than 12 segments in that time period. Larvae are defined here for convenience as specimens with 6 or less segments, juveniles those with 7 to 12 segments, and adults those with 13 or more segments.

RESULTS

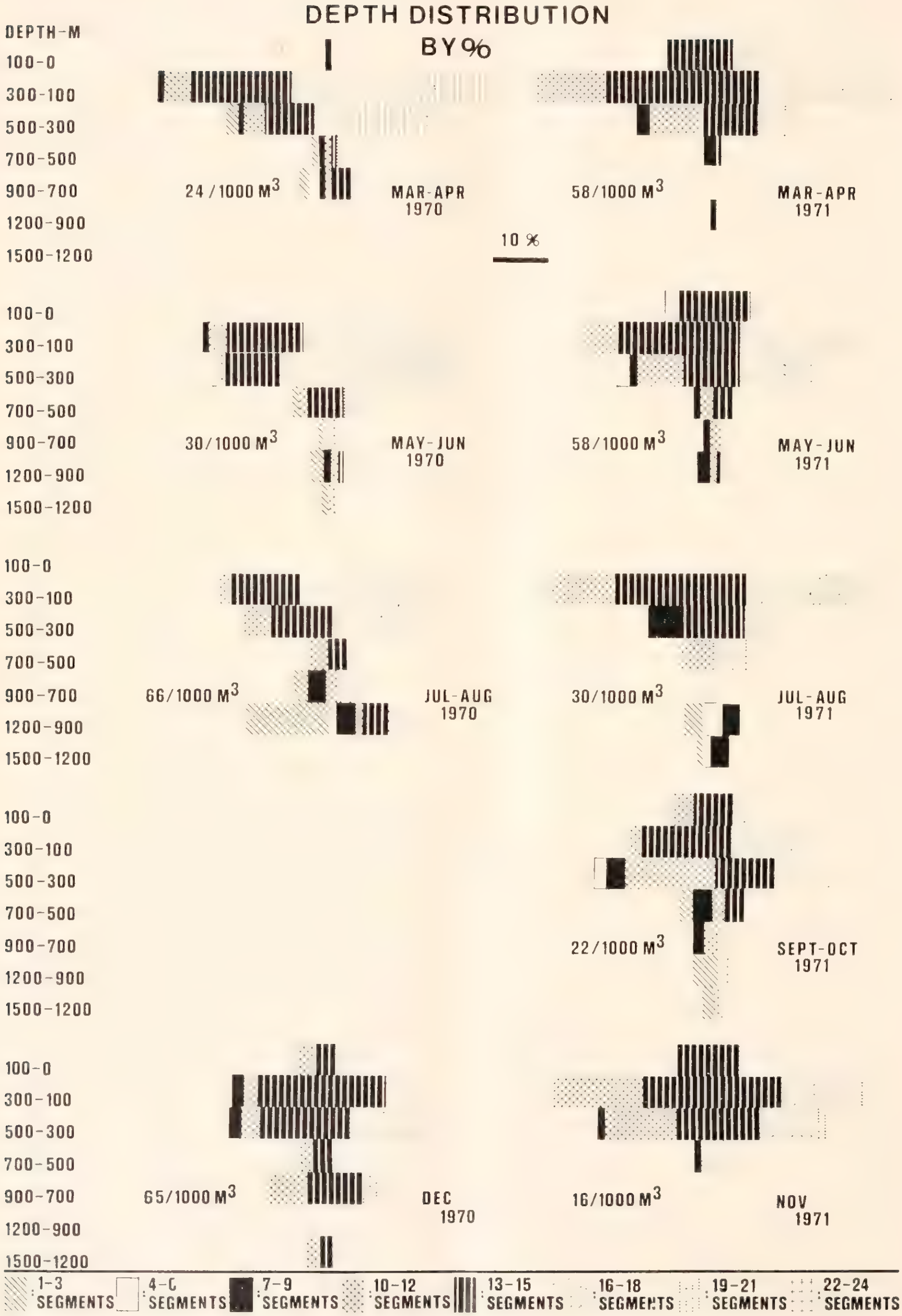
Vertical distribution and relative age

The vertical distribution of *P. longicirrata* changed seasonally as a function of the relative age of the specimens (Fig. 1). Animals with 13 or more segments were primarily found above 500 m where at least 60% of the total population was collected throughout the year in any given two month period. During the summer (and early fall) the percentage of animals under 500 m increased, reaching a maximum between July and October (Fig. 1). In contrast to those above, most of the individuals below 500 m had less than 13 segments. In fact, those with the fewest number of segments were found the deepest. For example, specimens with 1 to 3 segments were primarily found below 900 m and made up most of the population from 900 m to 1200 m (Fig. 1).

Density

The average density of animals from 0–1500 m fluctuated somewhat during the two year sampling period, but did so without demonstrating any apparent seasonal trends (Fig. 1). The mean density for 1970 and 1971 was 46/1000 m^3 and 37/1000 m^3, respectively.

The following is a list of the number of complete series of tows from 0–1500 m, the numbers of whole specimens (ws), and fragments bearing a prostomium (f) collected in a given two month period: March/April, 1970 (ws = 158, f = 30, 13 series from 0–1500 m); May/June, 1970 (ws = 71, f = 21, 5 series from 0–1500m); July/August, 1970 (ws = 73, f = 53, 4 series from 0–1200 m); December, 1970, (ws = 54, f = 8, 1 series from 0–1500 m, and 1 series from 0–700 m); March/April, 1971 (ws = 90, f = 14, 3 series from 0–1500 m); May/June, 1971 (ws = 106, f = 28, 4 series from 0–1500 m); July/August, 1971 (ws = 38, f = 52,


DEPTH DISTRIBUTION
BY %
DEPTH-M
100-0
300-100
500-300
700-500
900-700
1200-900
1500-1200
24/1000 M3
MAR-APR 1970
58/1000 M3
MAR-APR 1971
10 %
30/1000 M3
MAY-JUN 1970
58/1000 M3
MAY-JUN 1971
66/1000 M3
JUL-AUG 1970
30/1000 M3
JUL-AUG 1971
22/1000 M3
SEPT-OCT 1971
65/1000 M3
DEC 1970
16/1000 M3
NOV 1971
1-3 SEGMENTS
4-6 SEGMENTS
7-9 SEGMENTS
10-12 SEGMENTS
13-15 SEGMENTS
16-18 SEGMENTS
19-21 SEGMENTS
22-24 SEGMENTS

5 series from 0–1500 m); September/October, 1971 (ws = 47, f = 6, 4 series from 0–1500 m); and November, 1971 (ws = 17, f = 3, 2 series from 0–1500 m).

Occurrence of oocytes

With the hope of defining the breeding season and the development of sexual maturity in *P. longicirrata*, the occurrence of oocytes throughout the sample population was investigated. Oocytes as viewed through the body wall with a compound microscope were opaque, either spherical or ovoid, and contained a large amount of yolk. In general they were similar to the oocytes of this species shown by Reibisch (1895).

Oocyte diameters ranged from 8 μ to 120 μ, but most were between 20 μ and 40 μ. The ratio of the yolk diameter to the total oocyte diameter decreased from 0.7 in oocytes with a diameter of 20 μ to 40 μ to about 0.4 in the 60 μ to 120 μ range. These data, however, do not suggest at what size the oocytes are sexually mature or even what diameter constitutes a maximum size for this species. Occasionally, the coeloms of the middle segments of specimens were densely packed with 30 to 40 oocytes per segment. In most specimens, however, the oocyte density was 6 to 10 per segment in each of 5 to 10 of the middle segments. Of the 104 specimens which contain a minimum of 4 oocytes in each of at least 3 segments, 82% had 15 to 20 segments. No oocytes were visible in specimens with less than 13 segments. Thus, on this basis, the breeding population is probably comprised of animals wth 15 or more segments.

As to seasonal variations in numbers, the peak occurrence of oocytes in both years was in the two month period of May/June when 30% of those with more than 12 segments bore oocytes (Fig. 2). The rapid decline in percentages from May/June to July/August suggests that spawning took place in early summer.

Developmental features concerned with food procurement

Specimens with more than 5 segments were found to have a mouth, anus, a continuous digestive tract, and a muscled pharynx which was often everted. Although these eversions were probably evoked during fixation of the specimens, they do indicate a capacity for eversion, a necessary prerequisite for feeding. On the other hand, the digestive tracts of animals with two or three segments were incomplete and appeared to be restricted to the anterior half of the body. Well preserved specimens of this size, very similar to the figure shown by Mileikovsky (1970), had a dense opaque area in the anterior half. Such an area could indicate the presence of yolk, which may play an important role in the survival of the larvae below 900 m. As an adult, *P. longicirrata* does not develop jaws on its proboscis which implies that it is not carnivorous.

FIGURE 1. The corrected annual vertical distribution of *P. longicirrata*. The specimens were placed into 8 size classes based on the number of segments. Each size class is coded by a different type of shading. The horizontal length of the bars is proportional to the relative number of specimens from each depth interval and in each size class in a two month period. The solid black line near the top of the figure is equivalent to a 10% contribution. The numbers on the lower left side of each two month distribution represent the average density from 0 to 1500 m for the two month period.

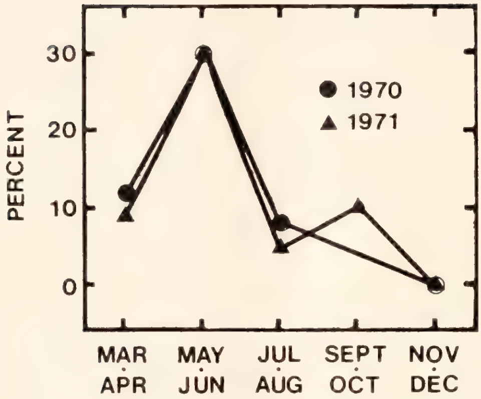


FIGURE 2. The percentage of specimens with 13 or more segments in a given two month period possessing a minimum of 4 oocytes in each of at least 3 segments. There are no data for the period of September–October, 1970.

DISCUSSION

Although it appears that *P. longicirrata* spawns during the late spring and early summer, it is doubtful that spawning is restricted to this period, as evidenced by the presence of some specimens with oocytes as early as March/April and as late as October (Fig. 2) and the occurrence of at least a few larvae for as much as 6 months of the year (Fig. 1). Since the breeding population is chiefly composed of animals living from 100 m to 500 m and very few ovigerous specimens were found below 500 m, spawning probably occurs above 500 m. At present nothing is known about fertilization or the subsequent fate of the spawners. It is possible, however, that females could either spawn more than once or shed the eggs or embryos over an extended period of time. According to Goodrich (1945) the Phyllodocidae, which at that time included the genus *Pelogobia*, possess a protonephromixium which generally provides a duct through which genital products can be shed. If *P. longicirrata* has such nephromixia, it is unlikely that the body wall would have to be ruptured and the female sacrificed to release the ova or embryos. However they are released, these ova or embryos evidently descend from the surface because most larvae are first found between 900 m and 1200 m. It is doubtful that the larvae feed at these depths; instead, they probably live on stored yolk retained from the egg. As they mature and slowly develop the morphology necessary for feeding, the juveniles move up in the water column. This upward movement occurs during and just after the yearly phytoplankton bloom in July and early August (English, 1961).

Although it was not appreciated at the time, the occurrence of the larvae below the adults may have been indirectly confirmed for *P. longicirrata* by two former studies. In the first, no larvae or juveniles were found during the summer in the upper 500 m of the Norwegian and Barents sea, although adults were numerous in this range (Mileikovsky, 1962). To explain this absence of larvae, Mileikovsky proposed that the adults reproduce at greater depths, implying that the adults and larvae should be found together at depths below 500 m. To test this hypothesis, two series of vertical tows were taken during August of one year in the Kurile-Kamchatka trench (Mileikovsky, 1969). Here adults were most numerous from 200 m to 1000 m and larvae from 300 m to 2000 m (Mileikovsky, 1969). These data were then interpreted as substantiating the above hypothesis that adults and larvae are found in the same depth range below 500 m (Mileikovsky, 1969). However, in light of the information now available from the Arctic ocean, the sampling program in the Kurile-Kamchatka trench may not have been sufficient to unequivocally delimit the vertical distribution of the larvae and adults. For instance, only two sets of tows in one month of one year were taken. Secondly, the lengths of the tows in the first set of samples covered too great a vertical distance. Thirdly, the number of animals listed for each depth interval in both series is uncorrected for differences in the lengths of the tows. Fourthly, it is not clear that the same number of tows was taken at each depth. Thus, the data from the Kurile-Kamchatka trench may not be inconsistent with the pattern present in the Arctic ocean, in which the larvae are found deeper than the adults.

On the other hand, it is possible that the vertical distribution of the larvae and juveniles relative to the adults may in part vary with the oceanic region in question in accordance with factors not yet appreciated, for there are some observations in the literature which are difficult to reconcile with the view put forth in this paper. For instance, young approximately 2 mm long were found in the same sample with mature adults in a 11 m tow taken near Hut Point, Antarctica (Ehlers, 1912). Friedrich (1950) also notes that samples with many individuals collected on deep tows in the Atlantic were either of just young animals or a mixture of young and mature, whereas no samples contained only adults. From these data Friedrich concluded that a small number of adults produce many offspring and that due to the negligible amount of turbulence they tend to remain together in the deep sea, and only slowly and passively disperse. Thus, although Friedrich confirms the presence of young in deep water, he apparently would disagree with the idea that the adults spawn in shallower water than that subsequently occupied by the larvae. In this case, however, any apparent contradiction cannot be resolved, because we do not know the exact size of the immature specimens and too few such samples were found by Friedrich to be certain that he presents a general case. On the other hand and in apparent support of deep living ovigerous individuals, three females with ova were collected between 750 m and 1250 m in the north Atlantic at two separate stations (Støp-Bowitz, 1948). However, only 5 specimens of this species in total were collected on the entire cruise and none of these were reported as immature. Another somewhat ambiguous picture of the vertical distribution of the adults versus the juveniles emerges from the work of Hardy and Gunther (1935) from South Georgia, Antarctica. From November to May the vertical distribution of the adults of *P. longicirrata* from 50 m to 1000 m tended to overlap the vertical distribution of the juveniles of all species of polychaetes collected. Although most of the juveniles were thought by the authors to be *P. longicirrata,* the fact that all species were lumped together in their data and that we again do not know the relative sizes of the juveniles precludes a critical evaluation of their results.

Finally, Tebble (1960) has found independent support for the contention made here that *P. longicirrata* is capable of seasonal changes in its vertical position.

Examining data collected by a vertical closing net on a transect following the Greenwich meridian from 70° S to 35° S, he determined that during the summer and early fall from November to April *P. longicirrata* was most abundant in the upper 100 m, but that during the winter it migrated into deeper water where it was fairly abundant throughout the year. Tebble speculates that its presence in the surface waters may be for purposes of breeding and feeding, although he presents no data to support these possibilities.

It is, of course, tempting to directly compare and discuss the vertical distribution of the adults of *P. longicirrata* with that found by others such as Tebble (1960), Gunther and Hardy (1935), and Mileikovsky (1962 and 1969) in different parts of the world. That temptation, however, is resisted for many of the reasons stated earlier in this paper. More productive comparisons can hopefully be made when the results on other groups of planktonic organisms collected in the same program from Fletcher's Ice Island T-3 become available.

In conclusion, the occurrence of the larvae below the adults is an interesting and even a bit unusual, because as Marshall (1954) notes, the larvae of most species are either found above or in the same range as the adults. With *P. longicirrata* one can presently only speculate on an explanation for this phenomenon. One possibility, however, is that living deeper may reduce predator pressure when the larvae are too young to feed and thus unable to exploit the surface waters.

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SUMMARY

During a two year period the vertical distribution of *Pelogobia longicirrata* (Polychaeta, Annelida) in the central Arctic ocean changed seasonally in conjunction with the relative age of the organisms. Adults with thirteen or more segments were primarily found throughout the year from 100–500 m. The peak occurrence of oocytes in the two month period of May/June was followed by a rapid decline in July/August, suggesting a spawning season in early summer or late spring. Larvae subsequently appeared below 500 m where specimens with one to three segments accounted for most of the animals collected between 900 m and 1200 m. Specimens with six or more segments were the youngest observed to have a mouth, anus, a continuous digestive tract, and a muscled pharynx, indicating that larvae smaller than these are probably incapable of feeding. They may instead live off of stored yolk retained from the egg. It is suggested that as the larvae mature they move up the water column and eventually join the adult population. The number of animals collected throughout the two years varied somewhat without demonstrating any apparent seasonal trends. The mean density from 0–1500 m was 46/1000 m^3 in 1970 and 37/1000 m^3 in 1971.

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ABSTRACTS OF PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY

Abstracts are arranged alphabetically by first author. Author and subject references also will be found in the regular volume index, appearing in the December issue.

GENERAL SCIENTIFIC MEETINGS

AUGUST 19–22, 1974

Effects of a Ca^{++} Mg^{++} ionophore (A23187) on cytokinesis in the squid. JOHN M. ARNOLD AND LOIS D. WILLIAMS-ARNOLD.

Ionophores are compounds that affect the transport of specific ions across biological membranes and cause changes in the intracellular availability of those ions. Cytokinesis in the squid blastoderm has been demonstrated to be the result of contraction of the microfilamentous band at the furrow bases. When ionophore A23187 (1–5 μM dissolved in dimethyl sulfoxide; embryos in Ca^{++} Mg^{++} free sea water) was pipetted directly onto the cleaving blastoderm the furrows were immediately affected. The furrows first became more distinct and gaps appeared at the junctions of the furrows. After approximately one minute the furrows became visibly less distinct and gave the appearance of relaxation. In some cases, after two minutes embryos were transfered into normal sea water and observations continued. Approximately twenty minutes after the treatment the furrows had increased in depth and length, extending beyond the edges of the blastodisc and encompassing up to one half of the entire egg. Furrows in the process of forming greatly accelerated in depth and length immediately after treatment. Subsequent cleavages were delayed and eventually prevented completely although the cells did become binucleate. In embryos kept in Ca^{++} Mg^{++} free sea water after treatment, cleavage continued after an initial delay and the embryos appeared fairly normal although some cells were binucleate.

It is postulated that the ionophore first makes Ca^{++} available to the contractile system of the cleavage furrow, then removes the Ca^{++} from the furrow bases causing them to become indistinct and to relax. The long term effect on embryos in normal sea water of greatly enhanced furrows is probably caused by a greater availability of Ca^{++} from the surrounding media because in the absence of external Ca^{++} cleavage continued. Apparently the ionophore-enhanced Ca^{++} transport remained functional and allowed an abnormal amount of Ca^{++} to accumulate from external sources, thereby interrupting the normal Ca^{++} flux during cytokinesis.

This work was supported by NSF grant GB 22604 and NIH grant EY 00179.

Membrane-associated carbohydrate-containing macromolecules of Arbacia punctulata. SUSAN J. ATLAS, JAMES D. JOHNSON, UTPALENDU S. MAITRA, AND ROBIN A. WALLACE.

Carbohydrate-containing macromolecules are apparently produced by the golgi apparatus and migrate from there to the cell surface. These molecules appear at the cell surface and are shed into the growth medium in both rapidly dividing cells and those not dividing at all.

This project was done to see whether membrane-associated carbohydrate-containing molecules were constant during early development of the sea urchin.

Samples of eggs, 3 hr embryos (32-cell stage) and 12 hr embryos (mesenchyme blastula) were homogenized in 2 ml of medium containing 0.5 M KCl, 15 mM EDTA, and 10 mM Tris-HCl, pH 8.0. A discontinuous sucrose gradient was constructed with the same buffer containing 1 mM EDTA. Gradients were centrifuged at 22,000 rpm for 2 hr. One ml fractions were collected from the bottom of the tube and measured for protein by absorbance at 280 nm.

Two clear membrane bands were observed, one at the 40%/35% interface, and the other at the 35%/30% interface. Fractions containing each of the bands were pooled and an eqaul volume of 10% TCA was added. The TCA precipitates were collected by centrifugation and washed once in distilled water. They were then dissolved in 2% SDS, 10% β-mercaptoethanol, 0.06 M Tris-HCl, pH 6.8 and electrophoresed in 8% polyacrylamide gels containing 0.1% SDS at 3 mAmp per gel.

The gels were fixed with 25% isopropanol, 10% acetic acid (which also removed SDS) and stained for protein with coomassie blue and for carbohydrate with PAS.

The protein pattern revealed with coomassie blue for the two different membrane fractions was quite different. The heavier, plasma membrane fraction contain many more bands than the lighter membrane fraction.

The carbohydrate stain showed no differences between egg, 3 hr, and 12 hr embryos. These results suggest that membrane-associated carbohydrate-containing macromolecules are constant during early development.

This study was performed at the Fertilization and Gamete Physiology Training Program at the Marine Biological Laboratory (NIH grant 3-TO1-HD00026–12S1).

Wound healing in the dogfish ocular lens. M. BAGCHI, C. HARDING, AND H. JAMPEL.

The ocular lens has proven useful as a model system for the study of wound healing. In the rabbit lens the epithelium responds to mechanical injury with a propagated wave of stimulation of DNA synthesis and mitosis which originates at the site of injury. Different species show different spatio-temporal patterns of response to injury. An analysis of the basis for these different patterns may help in better understanding the mechanism for turning the cell cycle on and off during healing. The advantages of the dogfish, among marine fish, include: after large injuries, the integrity of the eye and the transparency of the cornea are maintained, unlike some marine teleosts which may show progressive deterioration of the eye following injury. Also, we know more about the chemical characteristics of the aqueous humor (Doolittle, *et al,* 1958 *Biol. Bull.,* **115**: 335) than in most fish. This, combined with the large size of the eye, make it an attractive object of study. The eyes were injured by applying a freezing cone to the cornea for approximately ten seconds. The lenses were fixed at different time intervals through twenty days, and whole mounts of the epithelial layers were prepared. At all times studied, many of the cells in the wound area were elongated, and gave the appearance of extensive migratory activity. Thre is no evidence of mitosis, however, until six to seven days after injury. A high mitotic activity was evident throughout the remaining period studied. It appears, therefore, that cellular migration plays a prominent role in the wound healing process throughout the period studied. Mitosis, however, doesn't commence until the end of the first week, but then continues actively for at least the ensuing two weeks.

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Changes in surface antigens and surface enzymes at fertilization in sea urchin eggs. MARY LEE BARBER AND MICHAEL EDIDIN.

Arbacia punctulata egg surfaces treated with DTT and Pronase were studied immunologically using fluorescent antibodies and enzymologically in terms of glucose-6-P dehydrogenase (G-6-P-D). Treated eggs were serially incubated for 15 min with rabbit anti-jellyless whole egg serum and fluorescein conjugated goat anti-rabbit globulin. Stained eggs were fertilized and surface changes observed. Stain on normal egg surfaces goes into the fertilization membrane (FM). Stain on DTT-treated eggs, which do not make a FM, goes into the hyaline layer. This suggests that DTT treatment leaves a surface component on the unfertilized egg which becomes incorporated into the hyaline layer. Stain on DTT-Pronase-treated eggs becomes inhomogeneous but remains distributed over the entire surface of the blastomeres during cleavage, concentrating in the furrows, and fading as division proceeds. This suggests that fertilization and cleavage result in a rearrangement or incorporation of new material into the surface. G-6-P-D activity was found in egg ghosts and with lower activity on whole egg services. At fertilization, specific activity of ghosts increased almost five-fold, but on whole eggs decreased 10%. DTT treated whole eggs had little change in G-6-P-D specific activity but homogenates had 30% less than normal. DTT-Pronase-treated unfertilized eggs had 22% of normal activity, increasing to 46% after fertilization. Activity of whole eggs recovered with time. This suggests that the enzyme is on and continually released from the surface. DTT treatment removed surface material so that the FM is not formed, but leaves G-6-P-D activity and antibody staining material going into the hyaline layer. Pronase removes the enzyme activity and the material going into the hyaline layer, but it can be replaced after fertilization.

This study was performed in the Fertilization and Gamete Physiology Training Program at the Marine Biology Laboratory in Woods Hole (NIH grant 3-T01-HD00026-12S1).

High-affinity choline transport: uptake and metabolism of choline and pyrrol choline by synaptosomes from the optic lobe of squid (Loligo pealei). L. A. BARKER, M. J. DOWDALL, G. R. VICKERS AND T. W. MITTAG.

Cholinergic nerve terminals possess a unique high-affinity uptake system for choline (Ch), the immediate precursor of acetylcholine (ACh). Mixed populations of synaptosomes from mammalian brain take up Ch by both high (H) and low (L) affinity systems ($\mathrm{Kt_H} \simeq 1$ μM, $\mathrm{Kt_L} \simeq 50$ μM) but the subsequent acetylation of Ch to ACh by the enzyme, choline acetyltransferase (CAT), is associated only with the high-affinity system. The coupling between transport and acetylation is tight with a stoichiometry of $0.7-1$. Pyrrol-choline (N-methyl, N-hydroxyethyl pyrrolidinium) is a choline-analogue which mimics Ch at both transport and acetylation sites in rat brain synaptosomes. Synaptosomes from squid optic lobe are highly enriched in cholinergic nerve terminals and contain high and low affinity transport systems for choline. They thus provide an excellent model system for studying the generality of (a) the tight coupling phenomenon and (b) the mimicry of Ch by pyrrol-Ch. Uptake and metabolism of both [N-Me-^{3}H]-pyrrol-Ch and [N-Me-^{3}H]-Ch by squid synaptosomes was studied by incubation in Tris-buffered (10 mM, pH 7.4) artificial sea water at 26° for 5 min. Incubation was terminated by filtration through 0.65 μ millipore filters which were then used for determination of total uptake and molecular distribution of ^{3}H. Uptake of pyrrol-Ch and Ch were essentially the same: pyrrol-Ch Kt's (μM) $H = 6$, $L = 38$; Ch Kt's $H = 2-4$, $L = 38$: pyrrol-Ch Vmax's, $H = 7.4$ $L = 8.9$. These results are quantitatively (Kt's) and qualitatively (Vmax's) the same as for the rat. However the intraterminal acetylation of pyrrol-Ch revealed differences not previously seen in rat; namely the coupling between transport and acetylation had a stoichiometry of 0.3 for pyrrol-Ch and 0.5 for Ch (cf pyrrol-Ch = Ch = $0.7 - 1.0$ for rat). The kinetic parameters for solubilized CAT from squid optic lobe were determined using [^{3}H]-acetyl CoA and non-radioactive pyrrol-Ch or Ch: values were, Km's (μM) 6410 and 663, V max's (μmol/min/g original tissue) 1.9 and 12.9 for pyrrol-Ch and Ch respectively. Thus pyrrol-Ch is a relatively poor substrate for solubilized squid CAT as in rat (about 3% as effective as Ch in both cases). These findings indicate that there are subtle differences in the way squid cholinergic synaptosomes and rat cholinergic synaptosomes acetylate Ch and pyrrol-Ch *in situ*. These differences cannot be explained by the behavior of solubilized CAT towards these substrates.

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Interspersion of unique sequence DNA with repetitive DNA elements in the genome of Cerebratulus lacteus. THOMAS BARNETT AND R. TIMOTHY KOVACIC.

Previous studies on the sequence organization of sea urchin and *Xenopus* DNA's, both higher deuterostomes, indicate that the unique sequence DNA is interspersed with repetitive elements approximately 250 nucleotide pairs long. Current theories ascribe regulatory roles to those repetitive DNA elements adjacent to unique sequences. Because of the basic differences between protostomes and deuterostomes, it was of interest to determine whether any fundamental changes had taken place during the evolution of their respective genomes. For this reason, we undertook an examination of the DNA of the nemertean flatworm, *Cerebratulus lacteus,* whose phylum is placed close to the branchpoint of divergence of protostomes and deuterostomes. After purification from sperm, the DNA was sheared to two different fragment lengths and sized by alkaline sucrose sedimentation. Reassociation curves of both 300 and 2800 nucleotide pair fragments were determined by hydroxyapatite chromatography. Computer fits of the 300 nucleotide pair curve suggests three components of different reassociation rates, fast, moderate, and slow, each comprising about one-third of the total. Analysis of the 2800 nucleotide pair curve indicates that the reassociation rate is considerably enhanced over and above that predicted for simple length differences, since the reassociation was essentially complete well before the theoretical reassociation of the unique sequences. This suggests that the fast reassociation of the unique DNA is due to interspersion of unique DNA with repetitive DNA elements. Information as to the size of the interspersed repetitive element was provided by incubating 2800 nucleotide pair fragments, after denaturation, to a repetitive C_oT (C_oT 10), and subjecting this DNA to both hyperchromicity measurements and single-strand specific nuclease (S_1) digestion. The hyperchromicity of the C_oT 10 DNA indicates that approximately 24% of the DNA is in duplex form. Likewise, S_1 digestions of C_oT 10 DNA suggests that approximately 27% of the DNA is also duplex material. Therefore, about 25% of the DNA at this C_oT is in the form of repetitive sequences. Sizing experiments on the S_1-resistant duplexes show that a very large proportion of this DNA is in the form of about 250 nucleotide fragments, with a somewhat smaller proportion being larger than approximately 1000 nucleotide pairs. These results suggest that the DNA sequence organization (in *Cerebratulus*) is probably not qualitatively different from the organization found in higher organisms.

Internal potasium ions modify sodium channel selectivity. TED BEGENISICH AND MICHAEL CAHALAN.

One of the major problems in membrane biophysics is to determine the mechanisms by which ionic channels distinguish between different ionic species. Chandler and Meves (1965, *J. Physiol.*. **180**: 788) calculated sodium channel selectivity ratios for several cations and found that these ratios are unaffected by changes in pH, axon deterioration or inactivation. However, dilution of the internal KCl medium with isotonic sucrose produced an apparent increase in the selectivity for K^+ relative to Na^+. This change is selectivity as measured from changes in reversal potentials can be explained by a small chloride permeability. an effect of membrane potential itself on selectivity ratios, changes in the internal ionic strength, or changes in the internal K activity. Using perfused voltage clamped squid axons from *Loligo pealei* we have shown that the changes in internal [K^+] is the most important factor in altering the selectivity ratio, P_K/P_{Na}.

Changing the ionic strength of the internal solution with the impermeant cations tetramethylammonium or TRIS at constant K^+ acivity produced little change in reversal potential and hence in the selectivity ratio, P_K/P_{Na}. Replacing the external chloride by the larger isethionate ion also had no effect on P_K/P_{Na}. This and other experiments indicate that the ratio P_{Cl}/P_{Na} is less than 1/500, a value too small to affect the determination of P_K/P_{Na}. Changes in external sodium concentration from 440 mM to 110 mM produced an average shift of 30 mV in the reversal potential. This amount of shift indicates a change in the P_K/P_{Na} from 0.12 to 0.11 over this range of membrane potential. Therefore, potential itself has little effect on selectivity. However, decreasing K^+ activity at constant ionic strength greatly increased P_K/P_{Na}. For example, changing internal K activity from 335 mM to 42.5 mM produced a change in P_K/P_{Na} from 0.069 ± 0.007 (6) to 0.38 ± 0.03 (3). A plot of P_K/P_{Na} as a function of $1/a_K$ is linear, suggesting the interaction of K ions with a saturable site controlling permeability having an equilibrium constant of 750 mM. Inward sodium currents were little affected by changes in internal K^+. However, measurements of outward K^+ currents show deviations from the independence principle also consistent with a saturable site for P_K.

A comparison of predatory behavior among the naticid gastropods Lunatia heros, Lunatia triseriata *and* Polinices duplicatus. CARL J. BERG, JR. AND MARY E. PORTER.

Predatory behavior of three species of naticid gastropods was observed in the laboratory for animals feeding upon gastropods and bivalves. The predators bore through their preys' shells and devour the body tissues within. The position of the borehole reflects the behavior involved in prey capture, manipulation, and positioning for boring. Borehole position was measured with respect to the longitudinal (anterior-posterior) axis of gastropod shells and the circular distribution of holes around the shells. For bivalves, a system of coordinates originating at anterior and dorsal shell margins and expressed as percent of shell length and width was used.

Within each naticid species the snalis bore into distinctly different areas on each of their prey species. For example, *P. duplicatus* differs in its anterior-posterior distribution of boreholes when all combinations of *Mya arenaria, Mercenaria mercenaria,* and *Nassarius obsoletus* are compared, and in its dorsal-ventral distribution between the bivalves *M. arenaria* and *M. mercenaria.*

Significant differences are also found among the naticid species in the position in which they bore into the same prey species. Boreholes of *P. duplicatus* are distributed differently along the anterior-posterior axis of *M. arenaria* than those of both *L. heros* and *L. triseriate,* but there are no differences among the dorsal-ventral distributions. Similarly, boreholes of *P. duplicatus* are distributed differently from those of *L. triseriata* along both axes on *M. mercenaria,* and from those of *L. heros* on both axes in *N. obsoletus.* Boreholes of *L. heros* and *L. triseriata* do not differ in distribution on *M. arenaria.* All these variations result from differences in prey manipulation and boring among naticid species.

It was shown that these differences may be used to separate and assess the effect of predation by each naticid species upon other mollusks in their habitat.

Inhibition by molybdate of Prasiola *cell attachment and completion of the* Chlorella *life cycle.* SCOTT E. BINGHAM, JEROME A. SCHIFF AND J. RAMUS.

Cells released by homogenization from the thallus of *Prasiola stipitata* Suhr attach to substrates and develop into mature plants. Attachment appears to involve secretion of a highly acidic, possibly sulfated, polysaccharide. Molybdate, which is known to block formation of adenosine 5′-phosphosulfate and, consequently, adenosine 3′-phosphate 5′-phosphosulfate the sulfate donor in sulfate esterification reactions of many systems, blocks attachment of *Prasiola* cells when administered in calcium and sulfur free media. Molybdate (0.3 M) inhibits completely; however, if within 24 hours molybdate is removed and sulfate added, cells recover the ability to attach. Sulfate added to prior to, or simultaneously with, equimolar molybdate allows 20–30% attachment, reflecting the competition of sulfate and molybdate. Molybdate added to attached cells results in a 40–50% release from attachment, indicating a possible turnover of polysaccharide after attachment. Sulfur starvation halts the *Chlorella pyrenidosa* Chick life cycle during cell enlargement prior to division. Addition of a utilizable sulfur source allows synchronous cell enlargement and division beginning 40 hours later. Since mutants blocked for sulfate reduction will not complete the cycle on sulfate, and since cycloheximide inhibits. protein synthesis is required. In order to test whether the formation of oxidized sulfur compounds (such as the sulfate esters) is required, molybdate was used to block reutilization of sulfate formed from oxidation of reduced sulfur sources in the mutants blocked for sulfate reduction. Molybdate (0.1 M) added 24 hours prior to methionine blocks completion of the cell cycle indicating the indispensability of oxidized sulfur for this process. Addition of sulfate to these blocked cells releases the inhibition and allows cell cycle completion suggesting molybdate is acting the ATP sulfurylase rather than complexing directly with polysaccharides. Taken together these experiments indicate a requirement of sulfated polysaccharides and, perhaps, other oxidized sulfur compounds for *Prasiola* cell attachment and completion of the *Chlorella* life cycle.

Differential labelling of Fundulus hemoglobins using different hemoglobin precursors. THOMAS A. BORGESE AND ROBERT CARTICA.

Electrophoretic patterns of hemolyzates obtained from various species of fish indicate the presence of multiple components. *Fundulus heteroclitus* and *majalis* both contain four hemoglobins separable by polyacrylamide, disc-gel electrophoresis. Hemoglobin I (Hb I) is the slowest and IV the fastest moving anodal components and together comprise nearly 30% of the total hemoglobin. Since different mechanisms might, reasonably, give rise to the different components, including aggregation-dissociation, oxidation of the heme iron, combination of the polypeptide chains with one another in different ways to produce additional components, or different rates of *in vivo* synthesis, we investigated some of the properties of these hemoglobins including *in vivo* and *in vitro* synthesis using ^{59}Fe (as the heme precursor) and ^{14}C-hydrolysate (as the globin precursor). Although Hb I is a minor component, it invariably has the highest specific activity with ^{59}Fe *in vivo* and *in vitro* in the presence of a high phosphate buffered medium. In contrast, and *in vivo* study of ^{14}C-labelled fish, over a period of 7 days at 18–22° C showed that in virtually every case Hb I specific activity was the lowest. By day 7 the specific activity of Hbs II, III and IV, expressed as per cent of Hb I was reduced to 130,140 and 100% respectively and seemed to be approaching unity. Evidence from SDS gels and analytical ultracentrifugation of fresh unfractionated hemoglobin indicated monomer and tetramer molecular weights corresponding to human hemoglobin. We suggest that ^{59}Fe labelling may not be indicative only of hemoglobin synthesis and that ^{14}C labelling may more accurately reflect hemoglobin synthesis in the short term. However, dissociation and reassociation of the hemoglobin chains might modify this interpretation and eventually lead to specific activity ratios of 1.00.

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Effects of ionophores on marine eggs and cation requirements for activation. Edward L. Chambers.

The divalent ionophores A23187 (0.7–50 μM) and X537A (20–50 μM), which have previously been shown to activate sea urchin eggs of other species (1974, *Biochem. Biophys. Res. Comm.*, **60**: 126, and 1974, *Proc. Nat. Acad. Sci.*, **71**: 1915), when added to suspensions of *Arbacia punctulata* eggs, cause expulsion of the cortical granules, elevation of the fertilization membrane, migration of the pigment granules to the cortex, secretion of the hyaline layer, dissolution of the egg nucleus, growth of a prominent monaster, and cleavage figures. Full activation also occurs when, after application of a suspension, the eggs settle on dried films of A23187 ($5\text{–}50 \times 10^{-4}$ μmoles cm^{-2}) or X537A ($5\text{–}50 \times 10^{-3}$ μmoles cm^{-2}), prepared by evaporation of ethanol solutions on plastic petri dishes. Activation by the divalent ionophores through monaster formation was observed in the eggs of *Echinarachnius parma*. In eggs of *Nereis limbata*, A23187 (5–10 μM) and X537A (25 μM) caused discharge of the cortical jelly-precursor granules with formation of a wide jelly zone, dissolution of the germinal vesicle, and first polar body formation the same as occurs following normal fertilization. The monovalent ionophores (up to 100 μM) dianemycin, dinactin, enniatin, monensin, nigericin and valinomycin failed to activate *Arbacia* eggs. A23187 caused *Arbacia* eggs to undergo the complete activation sequence in the absence of Ca^{++} and Mg^{++}, but in the absence of Na^{+}, as in choline substituted seawater or 0.53 M K^{+}, only expulsion of the cortical granules occurred. The addition of $1 - 5$ mM Na^{+} was sufficient to restore the full activation sequence. It is concluded that the divalent ionophores mediate the release of intracellular Ca^{++} stores which triggers (1) expulsion of the cortical granules and (2) activation of nuclear and cytoplasmic components. The latter process requires the presence of external Na^{+}.

Supported by NIH research grant HD-7213 and funds from the Florida Heart Association.

Facilitation of transmitter release at the squid giant synapse. Milton P. Charlton and George D. Bittner.

Facilitation of transmitter release was studied in giant and non-giant synapses in stellate ganglia of *Lolliguncula brevis* and *Loligo pealei* by means of intracellular microelectrodes. Transmitter release was evoked by pre-synaptic action potentials. Saline with manganese and reduced calcium was used to obtain post-synaptic potentials (PSP) under 5 mV (20° C). Salines were pH stabilized and oxygenated with 99.5% O_2 and 0.5% CO_2. Facilitation was measured during twin pulse and repetitive stimulation.

The time course of decay of facilitation in both species appeared to follow a dual exponential. In pre-synaptic giant fibers the early component of facilitation had time constants of decay between 4 msec and 10 msec. The later component of facilitation had time constants of decay up to 47 msec and appeared 15 to 20 msec after the first PSP in all cases. Maximim facilitation was 4.6 at zero time. In non-giant pre-synaptic fibers maximim facilitation was 0.3 to 1.1 while the initial time constant was between 4 and 20 msec. The growth of PSPs and facilitation during repetitive stimulation was not inconsistent with a model in which successive synaptic events each added equivalent amounts of facilitation which decayed with the same time course.

When temperature was lowered, facilitation at any particular stimulus interval increased while the time constant of decay lengthened. Hyperpolarization of the pre-synaptic terminal eliminated the undershoot of the pre-synaptic action potential and reduced but did not eliminate facilitation. With greater hyperpolarization the first of a series of pre-synaptic action potentials was followed by a small long lasting depolarization such that successive action potentials were smaller. Facilitation still occurred under these conditions.

Supported by NRC and Grass Fellowships to M. P. C. and NIH grants to G. D. B.

Activation of a voltage-insensitive conductance by inward calcium current. William Clusin, David Spray and M. V. L. Bennett.

Skate electroreceptor epithelium consists of a single layer of interspersed receptor and supporting cells. Extracellular spaces are occluded by apical tight junctions which divide the receptor cell membrane into lumenal and serosal faces. The lumenal faces of the receptor cells can generate a synchronous 70 mV action potential when the epithelium is current clamped. This action potential is TTX-insensitive, but is abolished by perfusion of the lumen with Co. No delayed rectification occurs in the presence of Co. When the epithelium is voltage clamped, depolarization of the lumenal faces evokes early inward and late outward currents. The inward current is carried by Ca. It appears not to inactivate and is greatly reduced by perfusion of the lumen with 15 mM EGTA and 10 mM Ca. The reversal potential for the inward current (determined by extrapolation of the leakage) is also reduced by EGTA. As the reversal potential is approached, activation of the late outward current is slowed, delayed and finally suppressed. After perfusion of the lumen with EGTA, the late outward current is suppressed at a lower voltage, corresponding to the new reversal potential for the inward current. Prolonged tail currents, whose time course is insensitive to voltage always follow activation of late outward currents. Prolonged tail currents do not occur when the stimulus voltage exceeds the computed reversal potential for the early current. Stimuli producing early inward currents facilitate onset of late outward currents during subsequent stimuli. We believe that Ca ions acting on the inner surface of the lumenal membranes activate the late outward current. Large voltage stimuli that produce no late outward current presumably exceed the Ca equilibrium potential. We ascribe facilitated activation of late outward current to residual cytoplasmic Ca. A growing body of evidence suggests that K-permeability of many excitable membranes is increased by intracellular Ca. Our data provide evidence for a separate conductance mechanism which is insensitive to voltage and activated only by the presence of Ca in the cytoplasm.

Supported by a grant from the Epilepsy Foundation of America and by NIH training grant # 5T5 GM 1674.

The effects of strontium on the timing of miniature end-plate potentials. Ira S. Cohen and William G. Van der Kloot.

For twenty years it has been known that miniature end-plate potentials sometimes occur in bursts, in which there is a rapid, clustered spontaneous output of quanta. Since the appearance of these bursts was erratic, they were difficult to study. However, Dodge, Miledi, and Rahamimoff (1969, *J. Physiol.*, **200**: 267) discovered that in Ringer containing 7–10 mM Sr^{++} the frequency of bursts was increased sufficiently to allow an analysis to be undertaken.

We studied miniature end-plate potential timing at the frog neuromuscular junction in 2 and in 8 mM Sr^{++}. The intervals between events were more clustered than the Poisson expectation. The deviation from the Poisson prediction is greater in 8 mM than in 2 mM Sr^{++}.

We have proposed that the timing of miniature end-plate potentials can be best accounted for as a branching Poisson process: a random "primary" release has a chance of being followed by a series of "subsidiary" releases from the same section of the terminal. The parameters for the branching Poisson process can be calculated from ln-survivor curves. The timing of miniature end-plate potentials in Sr^{++} is well fitted by branching Poisson models. In Sr^{++} there is an increased number of subsidiary releases from each primary site. The frequency of primary releases appears to remain unchanged.

We have also investigated the increased miniature rate that follows the end-plate potential. The release occurring during the end-plate potential might be regarded as a primary release, and the following miniatures might represent subsidiary releases. In 8 mM Sr^{++} there is an increased number of following miniatures per quanta in the end-plate potential. This fits with the idea that there are a greater number of subsidiary events with a shorter mean interval in elevated Sr^{++}. In summary, the effects of Sr^{++} on miniature release can be accounted for by the branching Poisson model.

This study was supported by the Grass Foundation and by Grant 10320 from the USPHS.

Effects of increased intracellular pH-buffering capacity on the light response of Limulus *ventral photoreceptor.* J. A. Codes and J. E. Brown.

Zwitterionic pH buffers (1 M MOPS, pH after dilution 7.2, PIPES pH 6.3 and 6.9, MES pH 6.3; pH adjusted with KOH) were pressure injected through a micropipette into ventral photoreceptor cells of *Limulus*. To determine the volume of solution injected $H_2S^{35}O_4$ (about 1 μM) was included in the injection solution. After injection the ventral eye was partially digested and the radioactivity measured in a scintillation counter. The cell volume was taken to be not more than 0.5 nanoliter. A second intracellular pipette containing 3 M KCl monitored membrane voltage. The buffered-filled electrode was used to pass current and membrane currents induced by 20 msec light pulses were measured by a voltage-clamp technique. The sensitivity (light-induced current/stimulus intensity) was measured after 5 min dark adaptation before and after injection. After injections causing intracellular buffer concentration to increase by at least 30–200 mM the sensitivity was in some cases unchanged and in no case (out of 9 cells) did it fall by more than 0.4 log units. For all these injections the time-to-peak of the light-induced current became shorter by 6–16%. As a control, 1.3 M potassium isethionate was injected (85 to 200 mM); these injections also caused no change in sensitivity, but caused the time-to-peak to become, if anything, longer rather than shorter. These findings appear to rule out mediation of the light response by a change in intracellular pH and provide a means of dissociating changes in time scale from changes in sensitivity.

Supported by NIH grants EY-00834 and EY-00835.

Axoplasmic free magnesium levels, and magnesium ion transport in squid giant axon. Paul De Weer.

The free magnesium concentration in the axoplasm of the giant axon of the squid, *Loligo pealei*, was estimated by determining the effect of intracellular injection of various Mg-citrate buffers upon the sodium pump. The sodium pump is inhibited by excess magnesium as well as by lack thereof. The Mg-citrate buffer which resulted in no change in sodium efflux was in equilibrium with a free magnesium level of about 4 mM.

Efflux of micro-injected ^{28}Mg from squid giant axon proceeds with a rate constant of about 0.003 min^{-1} in 400–500 μm axons at $21 \pm 1°$ C. Ninety-five per cent of this efflux is inhibited by the injection of apyrase, and seventy-five per cent is inhibited by the removal of external sodium ions. Increased intracellular ADP levels do not affect Mg efflux nor its requirement of external sodium ions. The dependence of Mg efflux on external Na follows hyperbolic kinetics with a $K_m(Na)$ of approximately 160 mM. Increasing extracellular Mg concentrations reduce V_{max} for efflux, with little effect on $K_m(Na)$. Potassium ions have little, if any, effect on Mg efflux, suggesting that Mg transport is insensitive to membrane potential. Lanthanum ions reversibly inhibit up to 95 per cent of Mg efflux, with half-maximal inhibition occurring at *ca.* 0.4 mM La. Electrical stimulation results in an extra Mg efflux of less than 10^{-14} mole cm^{-2} (impulse)$^{-1}$.

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3 ^{125}*I Iodination and autoradiography of the plasma membrane of* Arbacia punctulata *eggs.* B. S. Dunbar, J. Johnson, and D. Epel.

The surface proteins of *Arbacia punctulata* eggs were iodinated with ^{125}I using the lactoperoxidase procedure in order to follow changes in membranes which accompany fertilization. This method allows labelling of surface proteins with little modification of structure such that eggs can be normally activated.

The vitelline layer was first removed with 0.01 M dithiothreitol (pH 8.5). A maximum of 0.2 ml eggs were added to 2 ml MBL artificial sea water containing 40 μCi ^{125}I in 0.1 N NaOH and 0.2 MBL lactoperoxidase (Sigma). Ten μl aliquots of 0.06% H_2O_2 were added at 2 minute intervals for 10 minutes and the cells were incubated for an additional 10 minutes. Samples were counted using Bray's scintillation fluid in a Beckman 250 scintillation counter. Calculations showed that 5% of the total ^{125}I added was bound to the eggs and that 5.5×10^5 molecules per egg were bound.

After iodination, eggs were washed and half were activated by the method of Steinhart and Epel with the calcium ionophore (A23187). The ionophore was used to avoid introduction of sperm proteins. Activated eggs showed a 26% (8%) loss of label to the supernatant sea water as compared to unactivated control eggs, indicating that surface proteins are lost following fertilization.

Iodinated eggs were fixed with paraformaldehyde-glutaraldehyde mixture, post-fixed with OsO_4 and embedded in epon-araldite for autoradiography. 0.5 μ sections were mounted on slides and dipped in Kodak NTB2 emulsion and developed after two days at 4° C. Autoradiography of unactivated iodinated eggs showed a heavy concentration of label on the surface of the eggs.

This iodination technique does label surface proteins of the egg without loss of viability of eggs and can be used for further biochemical analysis to determine the fate of the plasma membrane after activation.

This study was performed in the Fertilization and Gamete Physiology Training Program at the MBL (NIH-3T01-HD00026-12S1.)

Reflexes in the tailspine system of the horseshoe crab, Limulus polyphemus.

Douglas A. Eagles.

The tailspine of the horseshoe crab can move freely within the cone delimited by the margins of the socket. During normal movements the tailspine rarely contacts the socket margins, its position actively controlled by the eight large muscles inserting at its base. One of these, the ventral flexor muscle, is innervated by two branches of hemal nerve 16, one of which appears to be exclusively motor, the other exclusively sensory. The sensory nerve includes the axons of at least two separate populations of receptor neurons. Cells of one group are multipolar and their dendrites terminate near the insertions of the flexor muscle fibers onto tailspine apodemes. Some of these neurons fire as a result of the tension elicited by a minimal stimulus to the motor nerve. These cells also fire during lengthening and shortening of the passive muscle and during isometric active contraction. Anatomical and physiological considerations suggest that these neurons are series tension receptors. The other group of neurons is located more distally and consists of bipolar and tripolar cells located near the arthroidial membrane and within the distal nerve processes. These cells fire during various joint movements but not during isometric contraction. Tension receptor activity elicited by an isometric contraction results in a reduced spike frequency in the motor nerve, indicating central inhibiton of the exclusively excitatory tailspine efferents. It therefore appears that the tension receptors inhibit homonymous motoneurons, as in vertebrates and crustaceans. Because the joint receptors are heterogeous it has not been possible to define specific reflex effects.

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Anaerobic synthesis of bacterial luciferase. ANATOL EBERHARD.

The bioluminescent bacterium, *Photobacterium fischeri* strain 8265, when grown aerobically in a rich medium, produces the enzyme luciferase and the cultures give off light. The isolated enzyme requires reduced flavin mononucleotide, long-chain aldehyde and oxygen for *in vitro* light production. When grown anaerobically in a highly buffered medium with glucose as the carbon source and disodium dithionite as a reducing agent, the cells gave off no light whatever but still produced at least as much luciferase as when they were grown aerobically. The presence of luciferase was demonstrated by aerating an anaerobic culture and measuring the subsequent light emission. After five transfers of 0.1 ml of culture to 10 ml of fresh anaerobic medium the final culture produced 500 times as much light upon aeration as did the original culture. Since the luciferase present in the original inoculum had been diluted by ten orders of magnitude by the transfers, this finding clearly indicates massive *de novo* anaerobic synthesis of luciferase. The anaerobic synthesis of luciferase in the total absence of light production suggests that the luciferase must have some other important cellular function.

Effects of univalent (Fab) antibody fragments on the fertilizing capacity of sperm of the sea urchin, Arbacia punctulata. W. R. ECKBERG AND C. B. METZ.

Rabbit antibodies against sea urchin sperm, rendered non-agglutinating by papain digestion (Asp-Fab), prevent sperm from fertilizing eggs. The present experiments were designed to determine the step or steps in sperm-egg interaction which are blocked by antibody.

Asp-Fab had no effect on sperm motility. Examined microscopically, sperm motility decayed at the same rate after treatment with Asp-Fab, non-immune rabbit gamma-globulin (CFab), or sea water, and sperm longevity was the same in all three cases.

Asp-Fab-treated sperm failed to fertilize dejellied eggs (pH 5 sea water, 5 min) or vitelline layerless eggs (0.05% trypsin, 30 min to remove both jelly and vitelline layers). Evidently, antisperm antibody treatment prevents sperm from engaging in some essential reaction(s) at the egg cell membrane level.

Sperm binding to the egg may be one such essential reaction. Sea water or C-Fab-treated sperm bound to normal eggs, dejellied eggs, and vitelline layerless eggs, although binding was somewhat less in vitelline layerless eggs. Asp-Fab, on the other hand, inhibited binding in each case, although it did not effect jelly penetration. Such inhibition of sperm attachment or binding to the egg cell membrane can account for the fertilization inhibiting action of antisperm antibodies.

This study was performed in the Fertilization and Gamete Physiology Research Training Program at the Marine Biological Laboratory (NIH grant 3T01-HD00026–12S1).

Adaptation and spatial summation in rods from the toad retina. GORDON L. FAIN.

Intracellular recordings from *Bufo marinus* were used to investigate light adaptation and spatial summation in "red" rods. The onset of diffuse background illumination produced an initial transient peak of hyperpolarization, followed by a fast and then a slow decay of potential to a steady plateau level. The threshold of single rods in the presence of the background increased as the background intensity increased, the two being linearly related (following the Weber-Fechner line) for 3 log units. At backgrounds of about 5×10^5 incident quanta/rod-sec, threshold began to deviate from the Weber-Fechner relation, and backgrounds one log unit brighter completely saturated the rods.

Saturation of the rod response was not caused by inhibition from cones, since increment-threshold curves had the same shape for backgrounds at 505 and 605 nm. Nor was saturation produced by suppression of the response due to the steady plateau potential of the background, since this was always less than half the maximum hyperpolarization elicited from the dark-adapted receptor. Since even the brightest backgrounds bleached only a small fraction of the visual pigment, saturation may occur at some intermediate stage in transduction between bleaching and the production of membrane voltage change.

Spatial summation was measured by recording from rods stimulated with centered, concentric spots of varying radii. Sensitivity (measured from intensity-response curves) increased as spot radius increased, for spots up to 400 μm in radius. Since sensitivity increased by more than a log unit as spot radius was increased from 25 to 400 μm, the photocurrent of a single rod is apparently responsible for less than 10% of its sensitivity. The receptive fields of rods were not changed by moderate background illumination, suggesting that increases in acuity during light-adaptation are not caused by a reduction of receptor spatial summation.

Supported by the Grass Foundation and by an NIH Fellowship, #EY-54840.

Glycine transport in the intestine of the toadfish – effect of luminal application of ATP in vivo. A. Farmanfarmaian and Derek Barkalow.

Substances which reduce ATP production are known to inhibit active transport of organic solutes. *In vivo* studies in this laboratory on the absorption of amino acids and sugars by intestine have not supported the hypothesis of indirect coupling of transport to ATP via Na^+ or K^+ gradients. The present experiments were designed to test the possibility of direct coupling of ATP to glycine transport through interaction with the "carrier."

Glycine absorption in toadfish intestine shows saturation kinetics with K_t about 5 mM and J_{max} about 30 μmoles per g per hr at 20°. At glycine concentrations of 10 mM or 20 mM, uptake is linear with time for periods up to 60 min. Under these conditions addition of 10 mM ATP to the luminal fluid causes a 30% reduction in absorption rate. Statistical analysis showed significance at $0.05 > P > 0.02$.

These results suggest that ATP is capable of acting on the carrier at the luminal side in a manner like that postulated to occur in the cell. Such a mechanism might involve phosphorylation and conformational change of the carrier protein, leading to reduced substrate affinity. If this process occurs in the lumen when ATP is introduced, a reduction in lumen-to-cell transport would be predicted.

Supported by funds from the Rutgers Marine Sciences Center, Rutgers University, New Brunswick, N. J.

Intracellular Ca^{++} injection produces localized desensitization of Limulus *ventral photoreceptors.* A. Fein and J. Lisman.

Photoreceptors were penetrated with a microelectrode containing 0.09 M CaOH, 0.1 M Tris, 0.1 M EGTA (100–150 meg). The cells were dark-adapted until quantal responses were observed. Two microspots (nominal diameter, 10 μ) were focused on the photoreceptor; one centered on the electrode, the other 60–80 μ away. Spot intensities were adjusted so that 10–30 msec flashes evoked 20 mV responses. Small Ca^{++} injections (1–2 nAmp, 10 sec) produced a large (4–15 fold) attenuation of the response to the local spot while producing little (<1.5 fold) attenuation of the response to the distant spot. If the size of the injection was raised, the response to the distant spot was also attenuated. Following such injections, the response to the distant spot recovered faster (1–2 min) than the response to the local spot (4–5 min).

These results were found in cells where local and distant regions were of nearly equal (within 0.4 log units) sensitivity before injection. In some cells impaled with calcium electrodes, the region near the electrode was several log units less sensitive than distant regions and local effects of Ca^{++} injection could not be demonstrated.

In cells where a local effect was found, it was also possible to demonstrate that light-adaptation was local, as previously reported for this preparation by Fein. Lisman and Brown have hypothesized that a light-induced rise in intracellular Ca^{++} is important in regulating adaptation. The demonstration that both adapting lights and calcium injection produce localized effects is consistent with this hypothesis.

The results suggest that Ca^{++} can diffuse over neuronal dimensions, but that diffusion is sufficiently slow so as to produce intracellular gradients.

This work was supported by NIH grants EY 01362–01 to A. Fein and EY 01496–01 to J. Lisman.

The development of light production in the Annelid Chaetopterus pegamentaceus. GARY FREEMAN, GEO. T. REYNOLDS, AND ALAN WALTON.

In *Chaetopterus* larvae which have been raised at 22–23 degrees C, light production is first detected with a photomultiplier after KCI stimulation at 29 hours of development. During the next 10 hours of development the amount of light produced by a larvae increases 10 fold; the rate of increase in the amount of light produced as a function of time is uniform during this period. Image intensification studies show that the trochophore larvae has a mass of light producing cells on either side of its mouth. These cells are a part of the epithelium which covers the larvae. Cytological studies show that these cells contain many granules with a diameter of 2–3 microns; they closely resemble the photogenic cells of the adult. The spectra produced by the light from larvae and adults is the same.

If the AB and CD blastomers are separated at the 2 cell stage and raised in isolation the larvae derived from the CD blastomeres will produce light while those from the AB blastomeres will not. When compression is applied prior to the first cleavage the cleavage is frequently equal and the polar lobe material is segregated to both blastomeres. If the blastomeres are separated in eggs in which the first cleavage is equal as a result of compression and raised in isolation, both of the larvae derived from a pair of blastomeres will frequently produce light.

Supported by AEC grant DBER AT-11-1-3120 to G. T. R. and NIH grant GM 20024 to G. F.

Studies on the size distribution of loricae and the growth status of Tintinnida in Eel Pond. KENNETH GOLD.

The size distribution of loricae was used to identify the growth status of 3 species of Tintinnida in the plankton. *Tintinnopsis acuminata*, *T. dadayi*, and *Tintinnidium fluviatile* were each found to produce loricae having progressively shorter mean lengths, the changes often being detectable within a day or less. The very sudden shifts in the length-frequency distribution from longer to shorter loricae were interpreted as signalling the beginning of a period of rapid cell division. *T. acuminata* persisted in the plankton long enough to be studied throughout what is thought to be its entire bloom cycle lasting 6–8 days. Percentages of loricae in size categories of 5 μm intervals over the range of lengths were plotted daily, and the resultant family of curves then analyzed for events seen as changes in the slopes of the curves. Three phases were identified which have the following characteristics: (I.) An increase in the percentage of longer loricae in the population followed by their abrupt replacement with shorter individuals (1–2 days); (II.) the disappearance of the longer loricae from the plankton and an approximately linear increase in the percentage of shorter individuals; (III.) reversal of the trend set in phase II on the 6th day of the cycle. Based on similar curves for the smaller laboratory-reared species *T. parva* (2 μm intervals for the entire length range), these phases correspond—in batch culture—to the end of lag, exponential growth, and either stationary or death phase, respectively. The two other species are thought to have been from populations in phase I.

The following other Tintinnida were detected in numbers estimated as rare to abundant during June-August, 1974: *Stenosemella steini*, *S. oliva*, *Tintinnopsis minuta*, *T. rapa*, *T. baltica*, *T. platensis*, *Favella ehrenbergii*, and *Helicostomella subulata*.

Supported by a contract with the U. S. Atomic Energy Commission, reference number C00-3390-15.

Genome organization in the American oyster, Crassostrea virginica. ROBERT B. GOLDBERG AND JOAN V. RUDERMAN.

In deuterostomes a large fraction of the genome consists of 300 nucleotide (NT) long repetitive sequences interspersed with 1000 NT single copy sequences. A small fraction is made up of clustered repetitive sequences. The interspersed single copy length corresponds to that required to encode an average size protein. The interspersed repetitive sequences may play a role in the regulation of transcription of contiguous single copy structural genes (Davidson and Britten, 1973, *Quart. Rev. Biol.*, **48**: 565). It was of considerable interest to determine whether this pattern of DNA sequence organization extends to the protostomes as well. Our studies show that the arrangement of repetitive and single copy sequences in the oyster genome is similar to the deuterostome pattern, although there are some differences in the relative amounts of these sequences. Oyster DNA was sheared to 300 NT and 2600 NT long fragments. The 300 NT fragments were denatured, incubated to various C_oT (moles NT $\times$ l^{-1} $\times$ sec) values and assayed for per cent reassociation on hydroxyapatite. Computer analysis of the reassociation curve indicates that the oyster genome consists of 70% repetitive ($C_oT_{\frac{1}{2}} = 28$, complexity $= 0.18 \times 10^8$ NT pairs) and 30% single copy $C_oT_{\frac{1}{2}} = 850$, complexity $= 2.9 \times 10^8$ NT pairs) sequences. The repetitive sequences are present, on the average, 30 times per genome. 2600 NT fragments similarly analysed show 95% reassociation by C_oT 100, indicating that most 2600 NT fragments contain at least one repetitive element. Hyperchromicity measurements and S-1 nuclease digestion of 2600 NT fragments reassociated to C_oT 100 show that these fragments contain single copy sequences interspersed with repetitive elements. The A-50 agarose size analysis of the S-1 resistant regions demonstrate the presence of both 100–300 NT long repeated elements and clustered repeated elements. In comparison to the deuterostome genome, the oyster genome contains a relatively smaller amount of the 100–300 NT long repetitive elements and a larger amount of clustered repeated sequences.

This study was performed in the Embryology Course at the Marine Biological Laboratory.

Increase in conduction velocity of lobster giant axons during growth. C. K. GOVIND, F. LANG AND J. W. BLOOM.

Two pairs of giant interneurons may mediate the tail flip escape reflex in lobsters and crayfish. These are the lateral (LG) and medial (MG) giant axons running the length of the ventral nerve cord. In adult lobsters, conduction velocities of these giant axons range from 8–18 m/sec.

We have measured conduction velocities of MG and LG during postnatal development in the lobster *Homarus americanus* ranging in size from 11 mm (3rd larval stage) to 220 mm (sexually mature adult). In this growth series the conduction velocity of MG increased from 2.5 to 10 m/sec and LG apparently increased from 1.7 to 8 m/sec. In the early stages, conduction velocity of LG was not appreciably faster than the conduction velocity of other through conducting fibers in the ventral cord.

Preliminary studies were made correlating this increase in conduction velocity with increase in axon diameter. Cross section of the ventral cord above the first abdominal ganglion in a 14 mm larval lobster revealed a clearly identifiable pair of MG with diameters of 17 μ and several smaller (6 μ) axon profiles including one situated laterally. The conduction velocities at the corresponding stage is 3–5 m/sec for MG and 2 m/sec for smaller fibers.

This clearly indicates that giant fibers grow substantially during postnatal development and that this growth is associated with a corresponding increase in conduction velocity.

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Formation of dogfish lens fibers from soluble crystallins. G. GRIESS, S. ZIGMAN, AND T. YULO.

The relationship between soluble lens crystallins and the fibers of lens cortical cells was investigated. Aqueous homogenates of dogfish (*Mustelus canis*) were divided into total water-soluble (TSP) and water insoluble protein (TIP) by centrifugation at 3000 rpm. The TIP consists of fragments of individual fiber cells, as visualized in a light microscope (× 400). The protein associated with the cell surface maintains the characteristic cell morphology. The protein content of TIP relative to total lens protein is relatively high in the dogfish compared to mammals, and the fraction increases with aging.

Lens proteins compose a heterogeneous system, which was studied by combination of immunochemical and electrophoretic techniques. Rabbit antiserum was prepared against lens TSP using Freund's adjuvant. Purified TSP and TIP were dissolved and sulfonated with Bailey's reagent and then dialyzed. Parts of these solutions were mixed with TSP antiserum, and the precipitate formed was washed and then solubilized in 8 M urea-tris-glycine buffer, pH 8.4. Analysis of the makeup of these samples was by polyacrylamide gel electrophoresis with urea, and sulfonated TSP and TIP were references.

All components of the TIP could be identified with TSP components, as was further verified by the superposition of Ferguson plots (log mobility vs gel concentration) of these samples. A significant difference in molecular charge was found for only one of the components.

When the TSP components were sorted by DEAE-cellulose chromatography and stepwise selective membrane ultrafiltration using Diaflo filters, a preponderance of low molecular weight protein (<30,000 daltons) was found. This situation differs from mammalian lens proteins, which exhibit species with predominantly higher molecular weights. This fact may reflect the earlier evolutionary origin of the dogfish, and seems to indicate more embryonic forms of lens proteins.

The proteins associated with the fiber cell surface would affect the physical properties of the lens, such as deformability and dioptic power. The requirements for these properties in dogfish are obviously different from those in mammals, as is reflected by the lens protein profiles. It appears advantageous for dogfish to make fibers from soluble crystallins of lower molecular weight.

This work supported by PHS grant EY-00459; Monroe County Cancer and Leukemia Society.

Variation in corneal epithelial cell surfaces, as possibly related to cellular maturation and senescence (S.E.M. studies). C. HARDING, M. BAGCHI, S. SUSAN, AND H. JAMPEL.

The corneal surfaces of marine fish have been previously found to have numerous microprojections, which can best be seen with the scanning electron microscope. Five species of marine teleost were found to have a peculiar form of long curved ridges which are arranged concentrically. Two elasmobranchs, the dogfish and the skate, were found to have microprojections somewhat similar to those seen in the mammalian cornea (Harding, Bagchi, Weinsieder, Peters, 1973, *Biol. Bull.*, **145**: 438). In the present study, cell-to-cell variation in the pattern of microprojections has been analyzed in the smooth dogfish (*Mustelus canis*). Over the surface of an individual cell, the microprojections are typically uniform in appearance and distribution. From cell to cell, however, there may be significant differences in the microprojection pattern. The pattern varies primarily in the length and number of microprojections. Several distinct patterns can be discerned. For example, short numerous microvilli; numerous, relatively long microvilli; sparse, relatively long microvilli, as well as other patterns are found. These different types of cells can have an apparently random distribution. It is suggested that these different patterns reflect stages in the maturation and senescence of the surface epithelial cells.

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Theoretical analysis of amino acid uptake by toadfish liver. AUDREY E. V. HASCHEMEYER.

Studies on the kinetics of various processes associated with protein metabolism in toadfish *in vivo* have necessitated evaluation of uptake rates for amino acids entering the liver from the portal circulation. In the first few minutes after a pulse injection of an amino acid A (*e.g.*, ^{14}C-L-leucine) its movement may be described by a set of reversible monomolecular reactions representing the transfer of A from extracellular hepatic space (A_e) to intracellular space (A_i^{Free}) with rate constants k_1/k_{-1}, and to protein via a k_2 pathway, and the loss of A from hepatic space to the body and its return by a k_3/k_{-3} pathway. Values for k_3 and k_{-3} have been obtained from simultaneous measurements of disappearance of ^{3}H-mannitol (B_e) from liver after portal injection; k_2 is given by the rate of ^{14}C-leucine incorporation into protein relative to A_i^{Free} in 1-minute experiments. Saturation data for leucine uptake were used to obtain an estimate of the influx rate constant k_1. Various values of k_{-1} were then tested to determine the best fit of experimental data. The ratio of amino acid to marker substance in plasma draining from the liver (A_e/B_e) after hepatic portal vein injection of tracer quantities was plotted *vs.* total marker recovery in liver at the time of sampling (B_e) and compared with theoretical curves. Good agreement was found for ratios of k_1 to k_{-1} in the range of 4:1. This result suggests that a concentrative uptake process occurs at normal plasma leucine concentrations. A facilitated diffusion model ($k_1/k_{-1} = 1.5$) does not fit the experimental data.

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Spermiogenesis in Crepidula. CATHERINE HENLEY.

Mature spermatozoa of *Crepidula fornicata* and *C. plana* are normally polymorphic. The nucleated uniflagellated typical spermatozoon is apparently the functional male gamete. There are two size-classes of multiflagellated atypical sperm which, at the level of light microscopy, lack chromatin in the mature condition. One atypical type is much shorter and broader than the other. Spermatocytes from which typical spermatozoa develop are compact, with relatively little cytoplasm and with meiotic divisions of the usual type. Those giving rise to the two sizes of atypical sperm are larger and with disorganized meiotic figures. Atypical meiosis involves the loss of chromosomes from the spindle, after which they form cytoplasmic chromosome vesicles. These persist briefly in young atypical spermatids, in variable numbers, but eventually disappear.

Spermiogenesis of typical sperm begins with the extrusion of flagella from the syncytial multinucleated mass of spermatids. The nuclei condense from a diffuse condition (as seen in Feulgen preparations) to a very dense one, and mitochondria aggregate around the growing flagella. The spermatids continue elongation and often retain the syncytial condition until they are nearly full-grown. Development of the two kinds of atypical spermatozoa begins in both cases from a rounded compact cell, from which variable numbers of free flagella are extruded. At the side of the non-syncytial spermatid opposite that where flagellar extrusion occurs, a slender process appears and grows in length. Concurrently the flagella continue elongation and eventually become completely incorporated within the cell. So far as can be determined from light microscopy of living material and Feulgen smear preparations of testis, the two classes of atypical spermatozoa differ mainly in length and breadth, and in the relative abundance and size of refractile granular inclusions. The long slender atypical spermatozoa have fewer and smaller inclusions than the second atypical gamete.

Aided by a grant from the National Institutes of Health, GM 15311.

Bioluminescence in cell free extracts of the scale worm Harmothoë (*Annelida; Polynoidae*) ALBERT A. HERRERA, J. W. HASTINGS AND JAMES G. MORIN

Cell free extracts in 10^{-2} M phosphate, pH 6.6, were prepared by homogenization of magnesium anesthetized *Harmothoë imbricata* and *extenuata* collected locally. Classical 'luciferin—luciferase' tests were negative: cold and hot water extracts gave no luminescence upon mixing. However, luminescence lasting several minutes occurred upon addition of NADPH or NADH to cold water extracts. A factor augmenting this response occurs in hot water extracts. The NADPH response is sedimentable (50,000 × *g*, 1 hr.) while the stimulatory factor remains in the supernatant. Some alternative reductants (2-mercaptoethanol, ascorbate) were inactive, actually inhibiting the NADPH response. Reduced flavins were effective without exhibiting specificity, giving a flash lasting about 100 msec because of rapid autoxidation of the reductant. A similar but 100× more intense response was obtained with sodium dithionite, either by injection of oxygenated buffer to an extract previously reduced by excess dithionite, or by injection of dithionite into an extract containing oxygen. Using this latter assay we determined some properties of this material. It is sedimentable but, unlike the NADPH response, no stimulatory factor occurs either in the supernatant or in heated fractions. The involvement of only a single factor was confirmed by showing that activity was directly proportional to dilution. Activity occurs over a relatively wide pH range, optimal at 6.6, and is destroyed by heat. About 50% is lost in 5 min at 30° C; with a high activation energy. In summary we have isolated a particulate bioluminescent system whose *in vitro* activity is stimulated by reductants. Both NADPH and dithionite responses are low in extracts of scales which have been induced to luminesce prior to extraction. Neither activity occurs in bodies of luminous polynoids or in whole nonluminous polynoids. Finally, the activity does not appear similar to that reported for luminous annelids from other families, including *Chaetopterus* where the absence of polynoid-like activity was confirmed.

A study of the vitellogenic protein in the serum of estrogen-treated Ictalurus nebulosus. EILEEN D. HICKEY AND ROBIN A. WALLACE.

Fish oocytes may synthesize much of their yolk reserves as well as sequestering a yolk precursor, vitellogenin, from the serum. The yolk proteins are also more heterogeneous than those of other vertebrates. Studies of catfish vitellogenin were initiated to characterize it, and to determine its contribution to the total yolk proteins of the oocyte, and to their heterogeneity.

Vitellogenin synthesis was induced by intraperitoneal injection of estrogen (1 mg/50 g body weight). Five days later the animals were injected with ^{32}P sodium phosphate (50 μCi/100 g body weight) and the blood taken by heart puncture after 24 hours. Serum was chromatographed on DEAE-Cellulose in a citric acid-2-amino-2-methyl-1-propanol gradient. Vitellogenin was identified as a 280 nm-absorbing peak associated with ^{32}P activity and eluted at high ionic strength. This peak was absent in the serum of control male fish, and was present to only a slight extent in naturally vitellogenic females. Use of the proteolysis inhibitor phenylmethylsulfonyl fluoride (PMSF) in the sample and all buffers was required to elute the vitellogenin in a single peak with which all ^{32}P was associated. An acetone precipitate of this peak was analyzed by electrophoresis on 5% SDS-acrylamide gels. Both specific phosphoprotein and general protein staining revealed many bands. To determine whether this was the natural condition of fish vitellogenin or the result of handling, plasma samples from estrogen-treated and control male catfish were denatured and applied to gels immediately following collection in PMSF. No phosphoprotein bands appeared in the controls, and one major and a very weak minor band appeared in the sample from estrogen-treated animals, indicating that fish vitellogenin is probably a single high molecular weight protein as in other vertebrates.

This study was performed in the Fertilization and Gamete Physiology Training Program at the Marine Biological Laboratory (NIH grant 3-T01-HD00026-12S1), and supported in part by a faculty grant from Russell Sage College.

Genome sequence organization in coelenterate, Aurelia, *determined by DNA reassociation kinetics.* RATCHFORD C. HIGGINS.

Several deuterostromes have been studied in terms of their genome sequence organization. In order to determine the generality of these recent studies in animals, I have selected a similar study of a coelenterate, *Aurelia,* a representative of a lower phylum. DNA was obtained from male jellyfish gonads and from embryos. The native DNA exhibits a hyperchromicity of 28% and a t_m of about 83° C. Two preparations were sheared in the Virtis homogenizer and sizes were determined by alkaline sucrose gradient sedimentation to be 250 and 2000 nucleotides (ntp) respectively. These DNA's were separately melted and reassociated under controlled conditions. Hydroxyapatite was used to measure extent of reassociation. The 250 ntp fragments demonstrated two kinetic components, one 53% repetitive and the other 36% unique. The repetitive component was estimated to be present 170 times per haploid genome. The 2000 ntp fragments reassociated with kinetics similar to the repetitive component of the 250 ntp DNA. This suggests the unique sequences are adjacent to repetitive sequences.

SI single strand specific nuclease was used to destroy all but duplexes formed by repetitive sequences in reassociated unsheared DNA. These duplexes were then sized on agarose A50 columns showing two major size classes. About 40% of these duplexes are greater or equal to 2000 ntp and the rest are about 300 ntp. These observations are in accord with the emerging picture of animal genome structure throughout the animal kingdom.

Some aspects of transmission at synapses in the labyrinth of the toad fish. S. M. HIGHSTEIN, J. KEETER, M. V. L. BENNETT.

The posterior semicircular canal was removed from adult toad fish (*Opsanus tau*) and placed in a bath (167 mM NaCl, 2.5 mM KCl, 1.5 mM $CaCl_2$, 15 mM Tris or Pipes pH 7.3). Paired pipettes (100 μ tips) in the canal were used to pass current and record voltage across the sensory epithelium. Single afferent fibers were silent or spontaneously active at regular or irregular rates of 5–80/sec. Lumen positive (LP) transepithelial pulses which would depolarize the presynaptic faces of the receptor cells and hyperpolarize the lumenal faces increased the frequency of firing. Lumen negative (LN) pulses, which would hyperpolarize the secretory faces decreased spontaneous activity and caused off responses. Frequency-voltage curves were linear over a wide range but tailed off near zero frequency and reached a plateau of 60–300/sec at transepithelial voltages of approximately 200 mV. Larger voltages then decreased frequency. As LP pulses increased, latency of the first spike smoothly decreased to a minimum of 0.5–1 msec. Direct stimulation of the nerve near the sensory epithelium with wire electrodes evoked responses at latencies of less than 0.1 msec. Substituting Mg for Ca (1.5 mM) in the saline slowed or stopped spontaneous activity and shifted the frequency voltage relation towards higher voltages. We conclude from the irreducible latency and Mg inhibition that the hair cell synapse is chemically mediated. Transepithelial stimuli apparently act directly on presynaptic secretory membrane and in tonically active units ongoing transmitter release is increased or decreased by stimuli of appropriate polarity. Large LP pulses may be less effective because Ca entry into the hair cells is reduced.

Bath applied Noradrenalin 0.1–1 mM slows tonic activity markedly and shifts frequency voltage curves toward higher voltages. Electron microscopy reveals terminals containing dense core vesicles as well as afferent and efferent synapses. These results suggest a sympathetic innervation of the labyrinth.

Effects of external monovalent cations on ouabain inhibition rate of sodium pump in squid giant axon. ANN S. HOBBS.

Effects of external Na^+, K^+, and Cs^+ on the ouabain inhibition rate of sodium pump in giant axons from *Loligo pealei* were determined by following sodium pump activity (as ^{22}Na efflux) in the presence of various concentrations of these ions and 10^{-7} M ouabain. Relative pump rate was also computed, as the ratio of total ouabain sensitive efflux in the experimental solution to total ouabain sensitive sodium efflux in Na, 10 K ASW.

In sodium containing ASW, 5, 10, and 20 mM K^+ solutions gave relative pump rates of 0.59, 1.0, and 1.27 and rate constants (expressed as min^{-1}) for ouabain inhibition of 0.0596, 0.0484, and 0.025. When sodium was replaced by choline, 0, 10, and 100 mM K^+ solutions gave relative pump rates of 0.60, 1.6, and 2.04 and rate constants for inhibition of 0.0141, 0.0072, and 0.0036. Raising [Cs^+] in sodium ASW from 25 to 75 mM produced an increase in relative pump rate from 0.45 to 1.06, but only a slight drop in the ouabain inhibition rate constant: from 0.0517 to 0.0474. In choline ASW neither relative pump rate (1.65) nor ouabain inhibition rate constant (0.022) changed significantly when [Cs^+] was raised from 25 to 150 mM.

In both sodium and choline ASW, K^+ appears to slow the ouabain inhibition rate. Sodium, conversely, enhances the rate. For equivalent pumping rates, sodium produces about a four-fold increase in the ouabain inhibition rate. In the absence of Na^+, K^+ solutions have less than half the rate of ouabain inhibition as Cs^+ solutions showing the same relative pump rates. The fact that these differences are seen even at equivalent pump rates implies that this is more than a difference in the occupational state of pump stimulatory sites. It could imply that the sites affecting ouabain binding are physically separate from the pump sites, or that the transport mechanism is different according to what particular ion is present externally.

Supported by the Grass Foundation.

Analysis of fast axonal transport in the sonic motor nerve of the toadfish. P. N. HOFFMAN, J. L. BARKER, H. GAINER AND R. J. LASEK.

The fast component of axonal transport was studied in the sonic motor neurons of the toadfish. Axonally transported polypeptides were labeled by the local injection of microliter quantities of either ^{35}S-methionine or a mixture of ^3H-leucine and ^3H-lysine into the region of the medulla containing the motor neuron cell bodies. In one series of experiments cells were labeled with either ^3H-fucose alone or in combination with ^{35}S-methionine in order to label glycoproteins. Immediately after labeling, one sonic motor nerve was ligated near the point at which it enters the swim bladder, and the other nerve was left unoperated. Animals were sacrificed at various times after injection, and the radioactivity in 3 mm segments of the sonic motor nerves determined. A distinct peak of radioctivity was found moving at a rate of 70 mm/day. Radioactivity was found to accumulate in the region of the nerve immediately proximal to the ligature at times which were consistant with the rate of movement of the labeled peak. The absence of radioactivity in regions of the nerve distal to the ligature indicates that the local incorporation of blood-born precursors does not significantly contribute to the labeling of the nerve. Analysis of the labeled polypeptides of the fast component using SDS-polyacrylamide electrophoresis revealed the presence of a complex pattern of labeled polypeptides containing at least five major species with approximate molecular weights of 130,000; 60,000; 30,000; 18,000 and 5000 daltons. Electrophoretic analysis of the fast component material simultaneously labeled with ^3H-fucose and ^{35}S-methionine indicates that these major polypeptides are also labeled with fucose, suggesting that they are glycoproteins. These results are consistent with those of previous studies of the fast component.

Metabolism of cysteine in relation to the synthesis of isethionate by squid nerve. FRANCIS C. G. HOSKIN, MICHAEL L. POLLACK, AND ROBERT D. PRUSCH.

Isethionate, $HO\text{-}CH_2CH_2SO_3^-$, is the major anion of cephalopod nerve, occurring in squid giant axons, for example, at about 200 mM. A parallel has been noted in cephalopod nerve between the presence of isethionate and an unusual enzyme termed "squid nerve-type DFPase." Since DFP, $(C_3H_7O)_2\text{-}P(O)F$, is clearly not the natural substrate, it has been speculated that this enzyme may play a role in the presumed metabolic pathway from cysteine to isethionate. In order to explore this relationship we have examined several aspects of the metabolism of ^{14}C-labelled cysteine by squid nerve. For this purpose an improved radiochemical assay has been developed which involves the gas transfer of $^{14}CO_2$ out of the alkali in which it was trapped in Warburg vessels and into a scintillation fluid capable of absorbing 1 millimole CO_2 per ml (preblend 3A80, Research Products International).

The results show that, of the cysteine entering squid axons (240% of equivalent distribution in 1 hr from an external concentration of 10^{-5} M), 30% is decarboxylated and only 1% is further and completely degraded to CO_2. Carbons 2 and 3 of the other 29% are retained or metabolized in a different manner. Pyruvate, a somewhat similar 3-carbon compound, is metabolized quite differently by squid nerve. First, it is taken up at a much lower rate than cysteine (25% of equivalent distribution). Secondly, of the pyruvate entering squid axons, 100% is decarboxylated. Thirdly, 75% is further and completely degraded to CO_2.

Although we estimate that 3 to 5% of the cysteine entering squid axons is metabolized to isethionate or isethionate intermediates, we have not yet been able to relate the so-called DFPase to isethionate synthesis. The techniques for evaluating these metabolic pathways should now be applicable to this problem.

This work was supported by NIH grant NS-09090.

Preliminary studies on natural decomposition of Fucus *in two marine environments.* R. DOUGLAS HUNTER.

Studies on the change in dry weight, ash and organic carbon were carried out on decomposing *Fucus vesiculosus* in summer 1974. Dry *Fucus* in mesh bags (1 mm^2 mesh size) was placed on the bottom of a rocky shore one meter under mean low water and another set of bags on the bottom of a salt-marsh tidepool. Bags were collected weekly from each environment. Oxygen concentration was 5.2 ml O_2/l at 21° C at the rocky shore site and 0.14 ml O_2/l at 25.6° C at the salt-marsh site at the depth of the mesh bags (4 cm under loose detritus).

The bag contents at both locations rapidly decreased in dry weight and carbon so that after one week of decomposition 57% of the dry weight and 50% of the carbon remained. This decline continued at the rocky shore site but stopped at the salt-marsh site with about 40% of the dry weight and carbon remaining. Ash content of the rocky shore samples increased from 25% to 35% in the first 16 days. Carbon per mg ash-free dry weight changed only slightly at both sites over the course of the experiment (44 days), however the salt-marsh samples were consistently higher than those at the rocky shore, suggesting differences in the nature of the decomposer organisms at the two sites.

In vitro experiments to measure short-term leaching of ash and carbon from dry *Fucus* showed that a rapid loss of both of these components occurred in the first 20 minutes followed by a gradual increase in ash and a slow decline in carbon.

Nitrogen analyses are in progress, but these results already suggest fundamental differences between the rates and nature of decomposition in *Fucus* at a rocky shore environment as opposed to a salt-marsh environment.

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Mitosis and nuclear morphogenesis in Barbulanympha. SHINYA INOUÉ AND HOPE RITTER.

Utilizing Ritter's successful culture of symbiotic flagellates of the wood roach *Cryptocercus punctulatus*, we have followed single *Barbulanympha* cells through division by combined polarrized light and differential interference microscopy. In *Barbulanympha* a persistent nuclear envelope assists in the interpretation of chromosome movement: as described by L. R. Cleveland *et al.* (1934, *Mem. Amer. Acad. Arts Sci.*, **17**: 180), kinetochores embedded in the nuclear envelope attach to astral rays and link chromosomes to the extranuclear spindle. Shortening of the astral "chromosomal" fibers: (1) draws the nucleus to the previously formed spindle, (2) assists in spindle envelopment by the nucleus and (3) results in kinetochore movement to the stationary centrosomes (anaphase A). Subsequently, chromosome sets separate further with their respective centrosomes while the spindle elongates several fold (anaphase B). During anaphase A the mid-portion of the approaching nucleus is blocked by the spindle as the outer margins are pulled around the spindle by shortening astral rays. The margins of the nucleus meet anteriad to the spindle and gradually form a seam parallel with the spindle axis. The resultant nuclear tube keeps the elongating spindle from splaying in early anaphaseB. The seam then opens as with parting lips at midpoint along the spindle; the resulting cleft enlarges towards both poles as well as perpendicular to the spindle axis. As the spindle and nucleus elongate, the cleft expands laterally then posteriorly around the spindle to finally complete karyokinesis. For any cell type we believe this is the first description of the process of karyokinesis accomplished through nuclear morphogenesis.

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Effects of subzero temperatures on eggs and embryos of Arbacia *punctulata.* S. C. JACKOWSKI AND R. A. WALLACE.

Eggs and early embryos of *Arbacia punctulata* were exposed to subzero temperatures and assayed for normal development by examination under the light microscope. Samples were suspended in sea water and cooled to −5.8° C, then seeded with ice. Ten minutes later samples were cooled to temperatures ranging from −10.5° to −27.0° C at a rate of 0.7° C/minute and were immediately warmed at a rate of 2.1° C/minute. Eggs were subsequently fertilized or embryos were cultured in sea water 22° C and scored for viability at the 4-cell or blastula stages, respectively.

After cooling to −5.8° C, viability of eggs decreased 30%; cooling to −10.5° resulted in a 98% decrease. Viability of embryos cooled to −5.8° C five minutes to 114 minutes after fertilization decreased only 4%. Embryos exhibited a differential susceptibility to lower temperatures dependent upon cell size and state: viability of 15-minute 1-cell embryos, 2-cell embryos, and 4-cell embryos decreased 43%, 76% and 22%, respectively, after cooling to −15.0° C whereas viability of embryos involved in first cleavage division and second cleavage division decreased 85% and 90% respectively. Similar patterns of susceptibility were obtained after cooling to −10.5° C and −17.0° C. Viability of embryos cooled to −27.0° C, regardless of cell size and state, did not rise above 5%.

These studies demonstrate that there is a cell cycle dependency in the susceptibility to freezing damage; in addition, they indicate that future research into the protection of embryos at subzero temperatures is feasible and should be initiated at the 4-cell stage.

This study was performed in the Fertilization and Gamete Physiology Research Training Program at the Marine Biological Laboratory (NIH grant 3-T01-HD00026-12S1).

Fertilization-associated changes in the plasma membrane proteins of Arbacia punctulata *eggs.* James D. Johnson, Bonnie S. Dunbar, and David Epel.

Ultrastructural studies on fertilization indicate that the plasma membrane is altered after fertilization; this change primarily results from the fusion of the membranes of the cortical granules with the plasma membrane.

To study egg plasma membranes after fertilization, the surface proteins were enzymatically iodinated as described by Dunbar, Johnson and Epel (*Biol. Bull.* abstract this issue). Only the egg surface was labeled as determined by autoradiography and loss of label following pronase treatment. The eggs were activated with the Ca^{++} ionophore A23187, and within 20 min of activation 27% of the labeled protein is released into the seawater. To further characterize the fate of the labeled membrane, homogenates of iodinated eggs were placed on discontinuous sucrose gradients. Only one radioactive peak was detected at 40% sucrose (w/v) in both activated and unactivated eggs. When eggs were activated with ionophore or fertilized sperm, (1) 27% of the labeled protein is released into the seawater, (2) 30% is found in the cell sap as a small molecular weight species (TCA soluble) and (3) approximately 40% remains associated with the membrane. When eggs were activated with 10 mM ammonia (no cortical granule breakdown) only the 27% loss to seawater was observed.

Ten major radioactive species were shown by SDS gel electrophoresis of labeled membranes from unactivated eggs, and only one major labeled component from membranes after activation of eggs.

These data reveal striking changes and reorganization of the plasma membrane accompanying fertilization. One change (27% loss to sea water) is associated with metabolic activation. Other changes are associated with cortical granule breakdown of membrane fusion during normal activation. These include reutilization of 30% of original labeled membrane proteins and possibly reorganization of the remaining protein components.

This study was performed in the Fertilization and Gamete Physiology Research Training Program at the Marine Biological Laboratory (NIH grant 3-T01-HD00026-12S1).

Fine structure and permeability studies of a rectifying electrotonic synapse J. S. Keeter, M. Deschênes, G. D. Pappas and M. V. L. Bennett.

In the first three abdominal ganglia of the crayfish, both the lateral septate and medial giant fibers form electrotonic synapses with the giant motor fiber of the third root. Junctional membrances of these synapses rectify (Furshpan and Potter, 1959, *J. Physiol.*, **145**: 289). When the potential in the lateral or medial giant fiber (presynaptic) is positive relative to the potential in the motor fiber (postsynaptic), junctional resistance is low. At rest, and when the motor fiber is more positive, junctional resistance is high; thus orthodromic transmission is favored over antidromic. Pre- and postsynaptic membranes are closely apposed at small regions, in contrast to the wider separation at chemically transmitting synapses (Stirling, 1972, *Z. Zellforsch.*, **131**: 31). Our studies on *Procambarus clarkii* show that the junctional separation is only 40–50 Å, similar to that demonstrated at gap junctions in septa between segments of the lateral giant fibers Septal gap junctions appear symmetrical in that pleomorphic vesicles are present in the cytoplasm on both sides. The rectifying junction appear asymmetrical, dense material is often associated with the postsynaptic junctonal membrane, and a few pleomorphic vesicles are often found presynaptically. We assign no function to the vesicles which are often found in the axonal side of non-rectifying axosomatic and axodendritic synapses. Intracellular injected fluorescein crosses septal gap junctions and a number of other non-rectifying gap junctions (Pappas and Bennett, 1966, *Ann. New York Acad. Sci.*, **137**: 495). We do not see fluorescein crossing the rectifying synapse in either direction, although electrical transmission remains intact. Relative impermeability to fluorescein is also found in some embryonic electrotonic junctions. The morpohological bases for these permebility differences remain illusive.

Activation of sea urchin sperm chromatin template for DNA synthesis by Ca^{2+}, Mg^{2+}dependent endonuclease present in oocytes of Arbacia punctulata. S. S. Koide, L. Burzio and Y. Tanigawa

A Ca^{2+}, Mg^{2+}-dependent endonuclease was partially purified by homogenizing washed oocytes in 25 mM Tris-HCl (pH 8.0), 2 mM MgCl with a Dounce homogenizer. The homogenate was centrifuged at 15,000*g* for 15 min. The supernatant was fractionated by precipitation with $(NH_4)_2SO_4$ between 0–50% and 50–85% saturation. The sediment was suspended in a medium containing 0.01 M Tris-HCl (pH 8.0), 30% glycerol.

Chromatin was prepared from sperms washed three times with a solution of 75 mM NaCl, 24 mM EDTA (pH 8.0) and suspended in 0.01 M Tris-HCl (pH 8.0). The sperm suspension was homogenized in a Dounce homogenizer. The homogenate was centrifuged at 15,000*g* for 15 min. The supernatant was designated as sperm extract. The sediment was suspended in 0.01 M Tris-HCl (pH 8.0) and the process repeated. The sediment was resuspended and layered over a medium containing 1.7 M sucrose, 0.01 M Tris-HCl (pH 8.0), 1 mM EDTA. The tubes were centrifuged in a SW 25.1 rotor at 22,000 rev/min at 2° for 4 hr. The sediment was resuspended in a solution containing 40% glycerol, 0.01 M Tris-HCl (pH 8.0).

The endonuclease activity was determined by measuring the amount of radioactivity solubilized from [^{3}H]DNA gel. The template activity of chromatin DNA synthesis was assayed by a standard method using *E. coli* DNA polymerase.

Oocytes contained high alkaline endonuclease activities. The activities in the fractions precipitated between 0–50% and 50–85% ammonium sulphate saturation were 3.64×10^3 units/mg of protein and 11.25×10^3 units/mg respectively. On the other hand, seminal fluid and sperm extract (0.01 M Tris-HCl fraction) possessed very low enzymic activities .

Sperm chromatin was a poor template for DNA synthesis. On incubation with partially purified oocyte endonuclease, the template activity was greatly enhanced. These preliminary results suggest that oocyte Ca^{2+}, Mg^{2+}-dependent endonuclease might play a role in the association of sperm and oocyte DNA subsequent to fertilization and might activate chromatin for DNA synthesis.

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Fish lateral vestibular neurons: electrotonic transmission from primary vestibular afferents, electrotonic coupling between vestibulo spinal neurons and identification of efferent cells to the labyrinth. H. Korn, C. Sotelo, N. Kotchabhakdi and M. V. L. Bennett.

Field and intracellular potentials were recorded in lateral vestibular nuclei of the toad fish, *Opsanus tau.* Vestibulo spinal neurons were identified by their antidromic spikes following spinal stimulation. In these cells, stimulation of the ipsilateral eighth nerve evokes two component EPSPs, the first of which is transmitted electrotonically from primary vestibular afferents as indicated by its short delay. The latency of this first component was 0.34 msec ($SD = 0.06$; $n = 34$); the time of arrival of the presynaptic volley was 0.28 msec ($SD = 0.08$; $n = 18$) as measured from the peak of the extracellular field potential, and 0.27 msec ($SD = 0.08$; $n = 43$) as measured in presynaptic fibers. Thus the computed synaptic delay was 0.06 msec, which is too short for chemical mediation. Spinal stimulation evokes graded depolarizations in vestibulo spinal neurons. The short latency (<0.02 msec) of these potentials suggests electrotonic spread of antidromic activity in neighboring neurons. Similar graded depolarizations were also recorded in primary vestibular afferents, therefore coupling of vestibulo spinal neurons appears to be at least in part by way of these fibers. In confirmation electron microscopy studies reveal club endings with numerous gap junctions on LVN somata and proximal dendrites. In some cells sitmulation of the ipsilateral nerve evokes antidromic spikes. The presence of LVN neurons which send their axons to the labyrinth was confirmed by their heavy staining with Procion Yellow following axonal electrophoresis. Graded stimuli applied to the eighth nerve evoke graded short latency depolarizations as well as long latency EPSPs; the former could indicate electrotonic coupling of the efferent cells or electrotonic transmission from primary afferents resulting in a short latency feedback loop. Electrotonic coupling of LVN neurons should reduce the latency of the responses to physiological stimuli, and also, as in other systems, may cause increased synchronization of postsynaptic firing.

The relationship of small mode miniature endplate potentials and quantal content of evoked responses in the stimulated frog neuromuscular junction. M. E. KRIEBEL, G. D. PAPPAS AND S. ROSE.

There are two classes of spontaneous miniature endplate potentials (MEPPs) which are readily observed in small edge fibers of the *sartorius* muscle (Kriebel and Gross, 1974, *J. Gen. Physiol.*, **64**: 85). In the unstimulated preparation, amplitude histograms show that about 4% of the MEPPs form a distinct mode about 1/7 that of the major mode (which may range from 2.4–4 mV). Prolonged nerve stimulation (10 Hz, 15–45 min) reduced most MEPPs to small mode size. During the course of stimulation, the evoked responses became smaller until they appeared to be multiples of the small mode MEPPs. Finally, EPP amplitude histograms had the same profiles as MEPPs. In unstressed preparations, we adjusted extracellular Mg^{++} (up to 5 mM) and decreased Ca^{++} (to $\frac{1}{2}$) until failure of evoked responses were observed with 1 Hz nerve stimulation. The affect of excess Mg^{++} was progressive on both the MEPP and EPP amplitudes. The proportion of the small mode MEPPs increased and the mean of the major mode decreased. When the percentage of MEPPs contributing to the major mode was relatively small, many stimuli failed to elicit EPPs and some EPPs were the same size as small mode MEPPs. It is not clear at this time what the relationship is between small mode MEPPs and EPPs in high Mg^{++} and low Ca^{++}.

The effect of D_2O on sodium efflux from the squid giant axon. D. LANDOWNE AND V. SCRUGGS.

Replacing 97% of the H_2O in the bathing sea water rapidly and reversibly reduced the efflux of ^{22}Na from the squid giant axon by about 35%. The effect appeared to be linear with respect to D_2O concentration. In potassium-free sea waters D_2O reduced the sodium efflux by only 5–10%. This is not because D_2O affects only the potassium-sensitive sodium efflux as is shown by injecting arginine into the axon, reducing the potassium sensitivity. In these axons D_2O produced a larger drop in sodium efflux than potassium free sea water. D_2O also reduced the efflux in potassium-free sea waters by about 40%.

The effects of D_2O were fairly well mimicked by lowering the temperature a few degrees. That is to say the sodium efflux into potassium-free sea water was less temperature sensitive than the efflux into normal sea water.

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Anchorage of cytoplasmic filaments to intramembrane particles in the plasma membrane and in the nuclear envelope: transduction of membrane receptor topography to the genome. D. S. LEE AND J. METUZALS.

Small blocks of rabbit brain from the lateral vestibular nucleus were isolated in 0.25 M sucrose, standard salt solution (Ishikawa *et al.*, 1969, *J. Cell Biol.*, **43**: 312), 20% glycerol and freeze-etched. In electron micrographs of fractures of Deiters' neurons, 60 Å filaments extend from the cytoplasm into the A face of the plasma membrane fracture where the filaments anchor with 80–150 Å intramembrane particles. A regular cross lattice pattern of arrangement of intramembrane particles with short 60 Å fiilament tails attached can be observed in plasma membrane fractures of unidentified cellular processes. In the cytoplasm of such processes filaments are arranged in a lattice of the same orientation. In fractures of the nuclear envelopes, 60–150 Å filaments extend from the nuclear pore complexes into the fracture faces of the nuclear envelope membranes and anchor to the intramembrane particles of these membranes. Intramembrane particles often border the nuclear pore complexes. Small blocks from the lateral vestibular nucleus were treated with glycerol, standard salt solution and HMM, fixed and embedded. In electron micrographs of thin sections of such blocks, numerous actin-like filaments, labelled by HMM, can be seen in unidentified cell bodies and processes. These observations extend the reported findings on neurofilamentous network in Deiters' neurons by Metuzals and Mushynski (1974, *J. Cell Biol.*, **61**: 701).

The neurofilamentous network establishes polarity through the anchorage to the intramembrane particles of the plasma membrane and of the nuclear envelope. Such a polarity may have a twofold significance. First, in maintenance at the outer surface of the neuron, of a specific receptor topography in relation to the spatial order of the genome in the nucleus. Secondly, in transducing changes in receptor topography in the plasma membrane to localized and specific transcriptional responses of DNA.

An experimental approach toward understanding the role of meiofauna in a detritus based marine food web. JOHN J. LEE, KENNETH TENORE, JOHN H. TIETJEN, CARMINE MASTROPAOLO.

The rate of detritus degradation was measured in model systems maintained in 3 chemostats. Each reaction vessel (surface area ~ 5×10^{-3} m²) contained ~160 g of sediment (~4% detritus) removed from the surface of a salt marsh at low tide. The sediment was carefully frozen and thawed 3 times to kill most of the indigenous organisms and was aseptically cut and placed in the bottom of the reaction vessel while frozen. Microflora dislodged from surface detritus in the marsh by agitation and filtered through 8 μM membrane filters (Millipore SC) was inoculated into each reaction vessel along with 0.5g ^{14}C labeled (0.05 μCi/g) *Zostera* detritus (ground to 120μ particle size). One reaction vessel served as control, the 2 others were inoculated with either *Capitella capitata* (~ 10,000/m²) or *Nephthys incisa* (~ 1000/m²). One reaction vessel also contained the following meiofauna: nematodes—*Chromodorine germanica* (~ 2.5×10^5/m²), *Monhystera denticulata* (3.0×10^5/m²), *Rhabditis marina* (2.0×10^5/m²); foraminifera—*Quinqueloculina seminulum* (5×10^3/m²), *Elphidium incertum* (5×10^3/m²), *Allogromia laticollaris* (1.0×10^4/m²); a ciliate, *Euplotes vannus* (1×10^6/m²); and an harpacticoid copepod—*Leptocheirus* sp. (5×10^3/m²). The flow rate in the chemostats was adjusted so that the sea water in the reaction vessels was replaced daily. Aeration was vigorous. In addition to the radioactivity measured daily in the overflow vessel, $^{14}CO_2$ was trapped in an additional vessel with saturated KOH. Under the experimental conditions, it took ~ 6 days for the microbial population colonizing the detritus to reach stable levels (as judged by ^{14}C turnover). After 6 days, under the experimental conditions tested (26° C) ~ 1/20 of the radioactivity was released from the detritus per day. Total community respiration was 3 × higher in the system with both meiofauna and *Capitella* than in bacterized controls; respiration in the system with *Capitella* alone was ~ 50% > controls. Bacterial populations were highest in the system with *Capitella* alone and lowest in the system with the meiofauna. No statistically significant differences in the radioactivity of *Capi-*

tella or *Nephthys* were discovered between those foraging alone or with meiofauna. It is possible that the specific activity of the labeled detritus was too low to detect such differences.

This research was supported by NSF grant GA 28454.

Aequorin study of the suppression potential in the squid giant synapse. R. LLINAS AND C. NICHOLSON.

Stellate ganglia of squid (*Loligo pealei*) were isolated and perfused in oxygenated artificial seawater (ASW). The pre- and post-fibers forming the giant synapse were impaled. Two electrodes were introduced into the pre-fiber at its terminal. One contained 100 mM TEA Br for current injection and the other aequorin in 200 mM KCl to record potential. The post-fibers were impaled in the immediate vicinity of the synapse. Electrophoretic injection of TEA for three to four hours into the pre-fiber blocked delayed rectification. Aequorin was then pressure injected for a protracted time and the Ca^{++} induced light emission accompanying single prolonged action potentials in the pre-fiber was detected with a photomultiplier. The light emitted increased with the duration of the spike and could be detected 10 to 15 msec after spike initiation. Following TTX-ASW (10^{-6}g/ml) graded synaptic potentials produced by increasing presynaptic depolarization could be correlated with the amplitude of the aequorin flash. With presynaptic depolarization, beyond 150 mV (on the average), a suppression of the synaptic release occurred which returned at the end of the depolarization. In a parallel manner, the aequorin response was at maximum during maximum transmitter release and was absent at the suppression potential level, reappearing at the end of the current pulse. These results demonstrate directly that synaptic transmitter release is accompanied by an intracellular increase of Ca^{++} concentration, and that the suppression potential is produced by a potential-dependent decrease of intracellular Ca^{++}. Two mechanisms can explain our results, (a) the suppression potential represents the Ca^{++} equilibrium potential (b) Ca^{++} conductance is inactivated by large membrane depolarizations. We favor the first mechanism since no large resistance increase was seen at the suppression potential in the pre-fiber.

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Effects on Arbacia *hatching enzyme of 5-hydroxytryptamine and N-benzyloxycarbonyl-L-glutamate.* GARY W. LOPEZ AND DENNIS BARRETT.

Deeb has reported that the hatching of the Mediterranean *Arbacia* at hatching stage is specifically prevented by 5-hydroxytryptamine administered at fertilization to 8-cell stage. Our attempts to repeat the effect reveal that in the American *Arbacia*, 5-hydroxytryptamine exerts instead a general retarding effect on development. We observed the half-time for the hatching process, in embryos incubated at 200/ml in millipore-filtered sea water at 23.5° C, as 6.6 hr, to an error of 0.3 hr, both in controls and in embryos treated from 0.5 hr after fertilization with 20 to 200 μg/ml of the inhibitor. Beginning at about hatching time, the inhibitor causes a general retardation, demonstrated by the extent of development at 30 hr of incubation. Controls were: full pluteus; in 20 μg/ml: early pluteus (with short arms); in 50 μg/ml: early pluteus; in 100 μg/ml: late prism; in 200 μg/ml: ovoid gastrula to early prism. Some delay in the hatching of the last approximately 30% of embryos in 100 or 200 μg/ml of inhibitor we attribute to this latter effect. Having carefully tried to duplicate the conditions of Deeb's experiments, we have no explanation to offer for our disparate results except species differences between the congeneric *Arbacia*.

The hypothesis that the hatching enzyme of *Arbacia*, like that of *Strongylocentrotus purpuratus*, is an endopeptidase with affinity for glutamyl-bonds, was tested by using blocked glutamate as an inhibitor. Crude hatching enzyme was prepared by ultrafiltering the supernatant above hatched blastulae; substrate was casein, with lysine residues ϵ-dimethylated to reduce background at pH 8; release of N-terminals was monitored by fluorescence after reaction with fluorescamine in acetone. *Arbacia punctulata* hatching enzyme digests modified casein with a K_M of 0.4 mg/ml; N-benzyloxycarbonyl-L-glutamate (carbobenzoxyglutamate) competitively inhibits the process with a K_I of 2.4 mM.

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Purification and properties of an Arbacia *sperm fertilization antigen.* UTPALENDU S. MAITRA AND CHARLES B. METZ.

Univalent antisperm antibody (Fab fragment), made by papain digestion of rabbit anti *Arbacia* sperm antibody, inhibits fertilization. Fab fragments evidently inhibit fertilization by binding to and blocking one or more sperm surface antigens essential for fertilization.

Sperm extracts (prepared by repeated freeze-thawing and centrifuging at 105,000 × g for 60 min) neutralized the inhibiting action of Fab fragments (Cordel and Metz, 1973). The extracts evidently contain the essential "fertilization antigen(s)." This antigen was purified by fractionation on DE-52 column (0 to 1.4 M NaCl gradient in 0.01 M sodium phosphate pH 7.5). Five pools were made from the column fractions. Although pool II and III contains detectable antigens, they cannot neutralize the effect of Fab fragments. Pool V completely neutralized the action of univalent antibody, while pool IV did do so only partially, probably from cross contamination by pool V.

Pool V was further purified by chromatography on DE-52 (0 to 0.7 M NaCl gradient). The protein thus prepared shows only one band upon polyacrylamide gel electrophoresis (7.5% native gel). The material stained with both PAS and alcian blue and hence is a glycoprotein.

In a preliminary attempt at localization, purified antibody against this antigen was isolated by immunoprecipitation. When specific antibody treated sperm were challenged with fluorescine labelled goat anti-rabbit antibody, only the sperm head fluoresced suggesting that the antigen is located in the sperm head surface. The protein had no effect on the eggs as observed under light microscope. The purified protein was labelled with ^{125}I using chloramine T. Purified ^{125}I-antigen bound to dejellied *Arbacia* eggs. The binding was partially blocked by Fab, while non-immune Fab had no effect.

This study was performed in the Fertilization and Gamete Physiology Research Training Program at the Marine Biological Laboratory (NIH grant 3-T01-HD00026-12S1).

Responses to low oxygen stress in relation to the ecology of littoral and sublittoral snails. ROBERT F. MCMAHON AND W. D. RUSSELL-HUNTER.

Six species were studied including the littoral species *Littorina littorea*, *Littorina obtusata* and *Littorina saxatilis* and the sublittoral species *Acmaea testudinalis*, *Mitrella lunata* and *Lacuna vincta*. Respiration rates in sea water with decreasing oxygen tension were recorded using oxygen electrodes before and for 25 hours after a five hour period of low oxygen stress. After such stress, *A. testudinalis* and *L. saxatilis* pay a large conventional oxygen debt on return to full saturation while *L. littorea* pays a somewhat smaller one. There was no evidence of any regular oxygen debt for *L. obtusata*, *M. lunata* or *L. vincta*. After five hours of low oxygen stress *L. littorea*, *L. obtusata*, *L. saxatilis*, *M. lunata* and *L. vincta* all show elevated oxygen uptake rates at all oxygen tensions and particularly at low oxygen tensions for at least 25 hours. However, after five hours low oxygen stress *A. testudinalis* showed little change in oxygen uptake rate with decreasing oxygen tension. The degree of elevation of oxygen uptake rate after low oxygen stress varied among the five species, and was least in *L. saxatilis* and greatest in *L. vincta*. Assessed as percentages of the prestressed rates at full saturation, the oxygen uptake rate at 10% oxygen tension (about 0.526 ml O_2/l at 760 mm Hg and 20° C) *before* stress for *L. saxatilis* was 3.0% and for *L. vincta* was 8.5%. After 25 hours return to full saturation from low oxygen stress the corresponding rates were 31.8% for *L. saxatilis* and 222.1% for *L. vincta*. In these five species, this increased oxygen uptake rate (which also occurs in the freshwater snail *Laevapex fuscus*) after return to fully saturated sea water from low oxygen stress appears to be a short-term acclimation of respiratory rate allowing the snails to maintain relatively high metabolic rates if low environmental oxygen tensions are encountered.

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Filamentous network of the axoplasm, as revealed by freeze-etching of the squid giant nerve fiber, in relation to actin, tubulin and myosin components. J. METUZALS AND W. E. MUSHYNSKI.

(*A*) Electron microscopy of freeze-etched squid giant nerve fibers reveals in the axoplasm curved, sinusoidal and ringshaped filaments of 20–200 Å in diameter, which are assembled into a continuous space network formation. The individual filaments are continuous with irregular dense tangles of 20 Å filaments and 50 Å granules. (*B*) Actin-like filaments can be identified regularly in electron micrographs of axoplasm freshly extruded in standard salt solution containing 400 μg/ml HMM and negatively stained with 1% uranyl acetate, pH 4.4 HMM-decorated filaments can be seen in thin sections of axons treated with glycerol, standard salt solution containing HMM, fixed and embedded. (*C*) SDS polyacrylamide gel electrophoresis was carried out on: (1) freshly extruded whole axoplasm; (2) axoplasm filaments segregated by uranyl acetate and extracted differentially in 2 M guanidine-HCl and in phenol; (3) muscle actin and myosin as well as protein standards run in parallel. Proteins in the molecular weight range of actin and myosin are present in the axoplasm. Myosin-like protein has been isolated from squid brain by the method of Pollard *et al.* (1974, *Anal. Biochem.*, **60**: 258) and an antibody against this protein has been prepared.

The ordered state of the filamentous network in the axoplasm may be maintained through interactions of its several protein components, such as filamentous protein (70,000 M. W.), actin, tubulin and myosin (215,000 M. W.). The network could be in a state of equilibrium between assembled and disassembled monomers of these proteins. Probably, a number of factors, such as Ca^{++} ions, may cause a shift of such an equilibrium state between the monomeric protein pool and the ordered state of the network.

A photoreceptor sensitivity paradox. B. MINKE, S. HOCHSTEIN AND P. HILLMAN.

It is becoming apparent that the invertebrate visual pigment, in contrast to the vertebrate, has a dark-stable (long-lived) metarhodopsin state. Early Receptor Potential (ERP) measurements have shown that the visual pigment of the barnacle lateral ocellus has two dark-stable states, rhodopsin and metarhodopsin, with spectral sensitivities having maxima at 532 nm and 495 nm respectively. Because in this case rhodopsin is much more sensitive to red light than metarhodopsin, and less sensitive to blue, it is possible to shift substantial amounts of pigment from rhodopsin to metarhodopsin and vice versa.

We have attempted to correlate the amount of pigment in the rhodopsin state, as judged from the ERP, with the sensitivity of the LRP. Intracellular recordings from *Balanus eburneus* lateral ocellus photoreceptors were made. The cell was adapted to a saturating amount of blue light, and then dark-adapted for between 5 and 25 minutes. The threshold of the LRP was then tested with weak blue or red lights. The procedure was repeated following saturating *red* light, the threshold to red and blue lights being tested after the *same* dark times. For each test flash (red or blue) and dark time, no differences in threshold between the blue and the red adaptation were found. Since the ERP measurements under similar adaptation conditions appeared to show changes of a factor of at least 30 in the population of the rhodopsin state this seems paradoxical. It unlikely that the ERP pigment is not that responsible for the LRP, because: (a) several investigators using different techniques showed that the LRP action spectrum matches well the action spectrum of rhodopsin measured by the ERP; (b) the induction of a prolonged depolarizing after-potential, which has the same ionic mechanism as the LRP, and its suppression or prevention, all almost certainly arise from the same pigment as the ERP.

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Interspersion of repetitive and non-repetitive sequence elements in the DNA of Spisula solidissima. GORDON PAUL MOORE AND WILLIAM CRAIN.

It has previously been shown that the genome of a number of deuterostomes contains short [about 300 nucleotides (NT)] regions of repeated sequences interspersed with longer (about 1000 NT) segments of unique sequences. Since none of the protostomes have been analyzed in such a fashion we have undertaken a study of *Spisula solidissima,* the surf clam, and our data suggest that this genome is organized in a similar manner. DNA was extracted, sheared and sized on alkaline sucrose gradients. Fragments 300 NT and 2000 NT were denatured and reassociated and the percent reassociation plotted against the C_oT (DNA concentration times annealing time). Computer analysis of the data showed two components, one repetitive (43%) and one unique (38%) for the 300 NT fragments. The 2000 NT fragments reassociated much more rapidly and showed one component. The rate constants for the repetitive component of the 300 NT fragments and for the 2000 NT fragments were 0.117 and 0.083 respectively. These values are equivalent. We conclude that each 2000 NT fragment contains an interspersed unit of repetitive DNA as well as unique sequences.

2000 NT and 300 NT fragments were denatured, renatured to a C_oT where only repetitive DNA could reanneal, then melted in the spectrophotometer with native DNA. The hyperchromicity of the fragments was 3.9% and 8.8% respectively while that of native DNA was 26.8%. The fact that hyperchromicity of reassociated fragments decreases with increasing fragment size indicates that there are single strand regions present in reassociated DNA and that the percent of these regions is greater in larger fragments. This is evidence of intersperison of repeat sequences. The length of the interspersed repeat was estimated on the basis of hyperchromicity and percent of the associated molecule resistant to single strand specific Sl nuclease. Our estimate for the length of the interspersed repeated unit is 200 ± 100 NT.

Development of spontaneous hypersynchrony in the hippocampal cortex of the bullfrog, Rana catesbeiana. F. MORRELL AND N. TSURU.

Bipolar stimulating electrodes were applied to the hippocampal cortex of one hemisphere in the unanesthetized. partially paralyzed bullfrog. Recording electrodes were placed adjacent to the stimulation pair and on the contralateral homotopic cortex. Electrical stimulation for 2 sec once per hour at current densities just sufficient to induce a brief after-discharge (AD) on first application led gradually to more prolonged AD involving, first, the side stimulated (1° focus) and, secondly, the opposite hemisphere (2° focus). After several hours it was noted that in the intervals between stimulations spontaneous, high voltage, paroxysmal electroencephalographic (EEG) spikes appeared in the 1° focus. Rarely at first and later quite frequently these spikes gave rise to evoked hypersynchonous potentials in the 2° cortex. Eventually the hypersynchronous discharges appeared independently in each hemisphere, a given spike being unrelated in time either to the electrical stimulus or to the occurrence of any other spike. There was a remarkable constancy of the wave-shape of the spontaneous hypersynchronous potentials which was specific to each animal.

The wave-shape coherence presumably reflects a high degree of anatomical order and functional organization in the neuronal network giving rise to it. Once established the properties of the network are largely intrinsic—the same electrical morphology as occurs "spontaneously" being elicitable by transcommissural synaptic input to the region. Since hypersynchronous potentials occurred as often as 5 per min and had amplitudes of 0.5–1 mV compared with normal background rhythms of 25–50 μV, they indicate an enormous change in energy production and a major transformation of the functional properties of the tissue.

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Elongation factor 1 of toadfish liver. JENNIFER B. K. NIELSEN AND A. E. V. HASCHEMEYER

A molecular basis is being sought for the changes in rate of polypeptide chain assembly in protein synthesis *in vivo* associated with temperature acclimation of toadfish. The present work confirms the elevation in liver aminoacyl transferase activity previously found in summer cold-acclimated fish, and establishes that the change occurs in elongation factor 1 (EF_1), the protein responsible for binding aminoacyl tRNA at the codon recognition site. Similar high levels of EF_1 are found in winter toadfish acclimatized to 7° C. Identification and quantitation EF_1 was made with two assay systems: the binding of ^{3}H-phe tRNA to ribosomes in the presence of poly U and GTP; and the polymerization of ^{3}H-phe tRNA to form polyphenylalanine in the presence of poly U, GTP, ribosomes and excess toadfish liver elongation factor 2. Highly purified ribosomes were obtained from rat and toadfish liver.

EF_1 from other eukaryotic sources has been found to occur *in vitro* in heavy and light forms with different specific activities. To determine whether such forms may account for shifts in activity levels *in vivo,* the distribution of EF_1 actvity from warm- and cold-acclimated fish has been examined. Purification has been carried out by pH 5 precipitation of the post-ribosomal supernatant, ammonium sulfate fractionation, and chromatography on Sepharose 6β and hydroxyapatite. Two active forms are eluted from hydroxyapatite at about 0.2 M and 0.3 M potassium phosphate, pH 6.8, at 30-fold and 60-fold purification, respecively, relative to post-ribosomal supernatant. Similar distributions were obtained in preparations from warm- and cold-acclimated fish.

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Association of divalent cations with membranes of squid giant axon. James L. Oschman and Betty J. Wall.

Electron microscopy reveals that opaque deposits are often associated with the cytoplasmic faces of plasma membranes when calcium is present in the fixative. The phenomenon has been demonstrated in a variety of tissues by several different laboratories. Either glutaraldehyde or osmium can be used as the primary fixative. Microprobe analysis has shown that the deposits are composed of calcium and phosphorus (Hillman and Llinás, 1974, *J. Cell Biol.* **61**: 146; Oschman, *et al.*, 1974, *J. Cell Biol.*, **61**: 156). In squid giant axon, deposits are present along axolemma, Schwann-cell membranes, synaptic membranes, and synaptic vesicles. Here we report that deposits are infrequent in extruded axoplasm, and that they do not occur in the "sheath" from which axoplasm has been extruded. The deposits could have been washed off during processing, but it is more likely that they only form in intact axons. Other divalent cations (Sr, Ba, Co, Mn, Mg) can also produce deposits in intact axons. The large number of calcium atoms per deposit (about 10^6), the lack of specificity for calcium, and the scarcity of deposits in isolated axoplasm and sheath all indicate that deposits do not localize individual membrane binding sites. Deposits do not seem to localize the axonal calcium binding protein isolated by Alemà, *et al.* (1973, *J. Neurochem.*, **20**: 681) since this protein is found in high concentration in the cell bodies where deposits are infrequent. The deposits resemble the product obtained when axons are reacted for ATPase localization (Sabatini, *et al.*, 1968, *J. Cell Biol.* **38**: 176). It has occurred to us that deposits may form when calcium entering the cell during fixation precipitates with intracellular phosphate released as endogeneous substrates (*e.g.*, ATP or arginine phosphate) are hydrolyzed by membrane phosphatases. Squid axons contain about 1 mM ATP (Hodgkin and Keynes, 1957, *J. Gen. Physiol.* **138**: 253). This is the same concentration of ATP used in the conventional Wachstein-Meisel ATPase incubation medium. The half-time for ATP turnover is short enough to enable substrate to be hydrolyzed before the enzyme is denatured by the fixative. All divalent cations form insoluble phosphates and hence can be used as trapping agents for phosphate. While other explanations have not been ruled out, the possibility that deposits localize membrane phosphatases fits with available data and provides a basis for further study.

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A computer program for evaluation of amino acid distribution in vivo. Roger Persell.

Two sets of simultaneous first-order differential equations have been used to describe the time course of movement of a radioactive amino acid among various compartments in the toadfish (*Opsanus tau*) following pulse injection into the liver through the hepatic portal vein. The first set concerns the time period of about 0.5 min from the beginning of injection, during which no significant portion of the pulse is lost from the liver; in the second set of equations terms are added to account for loss of amino acid from liver extracellular space to the rest of the body. A computer program of 25 statements has been written in the FOCAL language for numerical integration of these equations by the simple Euler method and for graphic display of the final quantities representing fractional recovery of original dose in the various compartments as a function of time to 3 min after injection. The approach is based on that of Moore and Ramon (1974, *J. Theor. Biol.*, **45**: 249). Initial values for all compartments are set within the program, and adjustable parameters including all the first-order rate constants and the time increment for the integration (usually 0.02 min) are supplied for each run. The program, as run on the PDP8/M computer at the Marine Biological Laboratory connected to a Tektronix 4010–1 cathode-ray graphic terminal, first displays the axes for the desired output. Curves representing the various compartments are generated as the numerical integration proceeds. Reproduction of the output is obtained with a Tektronix 4610 Hard Copy Unit. Analysis of a set of data for tracer ^{14}C-leucine movement in liver at normal plasma leucine concentration (about 0.1 mM) showed good agreement with computer-generated curves for the two time periods used to simulate the movement of the pulse.

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Exogenous acetylcholinesterase: effects on synaptic transmission at the neuromuscular junction of the frog sartorius. A. Politoff, A. Blitz, and S. Rose.

Incubation of frog *sartorius* neuromuscular junctions (NMJ) for several hours in Ringer's solution containing 0.5 mg/ml of electric eel acetylcholinesterase did not block synaptic transmission. During this period the preparation was tested by stimulating once every 30 sec at room temperature. However, repetitive stimulation at 12 or 20 Hz during 20 min followed by a rest period of 45 min in the presence of enzyme caused a great diminution or abolition of the response (both end plate potentials and muscle contraction). This effect also occurred in curarized muscle (1.2 mg/l), indicating that this effect is independent of muscle contraction. Esterase inactivated with paraoxon (E-600, an irreversible organophosphorus cholinesterase blocker) did not cause blockage of transmission. Neostigmine methyl sulfate at high doses greatly increased the response of the controls, but had little or no effect on the NMJ treated with active enzyme. Untreated NMJ's recover after a rest of 45 min, whereas treated NMJ's do not recover, even after repetitive washings to remove the esterase and periods of stimulation and rest. Untreated NMJ's, when stained for acetylcholinesterase activity (Karnovsky and Roots, 1964. *J. Hist. Cytochem.*, **12**: 219), showed abundant reaction product, while treated NMJ's did not react or reacted very poorly. Preliminary ultrastructural observations of NMJ's stimulated first in the presence of active esterase and tested later for block of transmission before fixation suggest that the endogenous esterase is absent from the postsynaptic membrane, but that there is now reaction product present at nerve terminals in close association with the presynaptic membrane.

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Correlation of changes in pigment content with photosynthetic capacity of seaweeds as a function of water depth. J. Ramus, S. Beale, D. Mauzerall, and K. Howard.

Light attenuation in the waters of Woods Hole was measured with a calibrated photometer equipped with spectral band filters. The depth required for a 10-fold diminution of light intensity was 3.5, 7.0, 5.5 meters respectively for red, green, and blue light.

The effects of light attenuation on pigment content and photosynthetic capacity were studied in the red algae *Chondrus crispus* and *Porphyra umbilicalis,* and the green algae *Codium fragile* and *Ulva lactuca.* Samples of each were attached to vertically-buoyed lines, and the lines set out in Great Harbor for 7 days. All species produced more photosynthetic pigments with increasing depth. The ratios of specific pigment contents for algae grown at 6 meters compared to one meter were: *Codium* (chlorophyll 1.5, carotenoids 0.5); *Ulva* (chlorophyll 5.4, carotenoids 5.6); *Chondrus* (chlorophyll 1.5, phycobilins 2.3); *Porphyra* (chlorophyll 2.1, phycobilins 5.7).

Photosynthetic oxygen evolution was measured *in situ* by lowering bottled samples to various depths (to 10 meters), and the dissolved oxygen measured subsequently with a polarographic electrode. The optically thick *Chondrus* and *Codium* showed no differences in photosynthetic rate between shallow and deep water adapted samples. However, with the optically thin *Porphyra and Ulva,* deep water adapted specimens photosynthesized at about twice the rate at all depths as did their shallow water adapted counterparts. These results suggest that the total optical density of the seaweed is more imporant than pigment content *per se* in determining the rate of photosynthesis.

Light saturation curves were obtained for oxygen evolution in incandescent white light. Absolute rate differences between deep and shallow water adapted plants at saturating intensities agreed with the above *in situ* measurements. When normalized to the maximum rates, the curves for deep water adapted samples of both *Chondrus* and *Porphyra* were identical to the curves of their respective shallow water adapted counterparts, whereas deep water adapted *Ulva* and *Codium* had greater photosynthetic capacity at lower light intensities than the corresponding shallow water samples. Adaptation to the ambient light environment by seaweeds includes both quantitative and qualitative changes in the photosynthetic apparatus.

This research was supported by the N.S.F. grant GB-37149 to the MBL. Experimental Marine Botany Program.

Turnover of synaptic vesicles in photoreceptor terminals of the skate. H. Ripps, M. Shakib and E. D. MacDonald.

Pieces of eyecup from dark-adapted skates (*Raja erinacea* and *R. oscellata*) were excised under infra-red light, and incubated for 2 hours in an elasmobranch Ringer solution containing 0.5 per cent horseradish peroxidase (HRP). Electroretinographic recordings in response to dim photic stimuli indicated that receptoral activity was maintained throughout the period of incubation. Electron microscopic examination of these dark-maintained preparations revealed a marked uptake of peroxidase into intracellular compartments (coated vesicles, cysternae, and synaptic vesicles) within the terminals of the photoreceptors. In a large sample of sections, between 10 and 45 per cent of the vesicles contained reaction product. However, the uptake of tracer could be blocked by a regimen of light adaptation which effectively suppressed all photic activity in the electroretinogram. If, during incubation in HRP, retinae were exposed at 10 min intervals to an intense 1 msec flash from a xenon discharge tube, the receptor terminals were almost completely devoid of peroxidase. Although reaction product clearly outlined the cell surface, indicating that substrate had penetrated the tissue, fewer than one per cent of the vesicles were labelled.

These findings provide further support for the view that vertebrate photoreceptors discharge transmitter in darkness, and that light decreases the release of this substance. We have been able to mimic the effect of light (*i.e.,* suppress the uptake of HRP) in dark-adapted retinae by adding magnesium to the bathing solution, suggesting that calcium is necessary for the release of synaptic vesicles from the receptor terminals. In addition, it seems likely that the incorporation of HRP into vesicles of physiologically active terminals reflects a mechanism for the retrieval of vesicle membrane following exocytosis. Perhaps more important, the peroxidase technique should prove particularly useful for tracing neural pathways in the vertebrate retina.

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Evidence for the synaptic vesicle calcium binding site at the neuromuscular junction of the frog sartorius. S. Rose, G. D. Pappas, M. Kriebel and A. J. Tousimis.

It has been shown that fixation of the neuromuscular junction of the frog *sartorius* muscle in the presence of 5, 50 and 90 mM $CaCl_2$ brings about the visualization of a single dense particle that is attached to or forms part of the synaptic vesicle membrane. In the same preparation, deposits were also observed inside mitochondria, at the triad region of the sarcoplasmic reticulum and on the postsynaptic membrane (Politoff, Rose and Pappas, 1974

J. Cell Biol., **61**: 818). Electron probe analysis of the presynaptic terminals which were fixed with $CaCl_2$ reveals characteristic X-ray energy for calcium while the controls do not. Our results, like those of others (Oschman, *et al.*, 1974 *J. Cell Biol.*, **61**: 156; Hillman and Llinas, 1974 *J. Cell Biol.*, **61**: 146), indicate that the electron dense deposits contain calcium. When divalent cations which act like calcium in depolarization secretion coupling (*i.e.*., Ba^{++} and Sr^{++}) are substituted in our procedure, a single dense particle is also seen. When Mg^{++} is substituted for Ca^{++} it is not visualized probably because of its low atomic number. However, Co^{++}, another inhibitor of evoked release, can be seen at similar sites as calcium. Our findings show that when the frequency of miniature endplate potentials (MEPPS) is greatly augmented (~100 fold), the calcium binding sites of the synaptic vesicles can no longer be demonstrated. However, deposits are observed as usual in mitochondria, sarcoplasmic reticulum but more intensely however in the postsynaptic membrane than in the controls. The measured decrement in MEPP amplitude may be due to deactivation of the calcium binding sites of the synaptic vesicle membrane. These results have led us to the hypothesis that this particle corresponds to a real biological structure, the vesicle membrane calcium binding site, whose function is to bind the calcium ions that enter the presynaptic process upon depolarization and that this binding is an essential step in transmitter release.

A large change in axon absorption during the action potential. W. N. Ross, B. M. Salzberg, L. B. Cohen, H. V. Davila, A. S. Waggoner and C. H. Wang.

Absorption changes of several dyes, applied externally, were evaluated for their sensitivity as indicators of membrane potential during neuronal activity. Giant axons from the squid, *Loligo pealei,* were cleaned and placed horizontally in a chamber on a microscope stage. The axons were dyed for 20 minutes in a sea water solution of the dye and then washed in sea water bubbled with argon. Quasi-monochromatic light passing through a 150 μm length of axon was focused on a photodiode and the change in absorption recorded during the action potential. With one dye, 5-[(3-γ-sodium sulfopropyl-2(3H)-benzoxazylidene)-2-butenylidene]-1,3-dihexyl-2-thiobarbituric acid, a close analogue of "merocyanine 540" (Eastman Organic Chemicals), the increase in absorption during a single action potential could be detected with a signal-to-noise ratio of about 20:1. This signal was much larger than the signals found thus far in measurements of birefringence, light scattering and fluorescence on the same apparatus. For this dye the absorption increased during the action potential for incident light of 550–600 nm and decreased for light of 450–550 nm. Fluorescence signals measured with the same dye showed increases at all excitation wavelengths. These observations are consistent with the dye existing in the membrane in both monomer and dimer forms, with the equilibrium shifting toward the monomer during depolarization. We hope that these larger signals will facilitate the development of an optical system for simultaneous recording of activity in many neurons.

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Patterns of aerial and aquatic respiration in relation to vertical zonation in four littoral snails. W. D. Russell-Hunter and Robert F. McMahon.

Discussing littoral zonation, it is physiologically appropriate to assess for each level of habitat the mean proportion of time in each semilunar tidal cycle of 354.4 hours when that particular zone is actually bathed by sea water. Four snails are discussed here: *Acmaea testudinalis,* is actually a sublittoral limpet which strays into the littoral (100% to 87% submerged); *Littorina obtusata* lives near low water of neap tides mainly associated with fucoid seaweeds (85% to 65%); *L. littorea* is the dominant snail of the midlittoral (70% to 25%); and *L. saxatilis* lives around the high water level of neap tides (18%) and occasionally somewhat higher (2.5%, corresponding to *Melampus*). The three congeneric intertidal species are all capable of active locomotion and feeding in air as well as in water. We have now determined oxygen uptake rates in air as well as in sea water at 5° C intervals from 5° C to 45° C using Clark-type polarographic oxygen electrodes.

In *Acmaea,* aerial O_2 uptake is between 1/2 and 1/3 of the aquatic uptake at the same temperatures, and both show a steady increase with rising temperatures up to the temperatures beyond which thermal death occurs. There is no obvious adjustment to higher temperatures in this or in other truly sublittoral snails. In *Littorina obtusata,* the "lowest" of our three intertidal congeners, aerial respiration within the normal temperature range shows a rise similar to that for aquatic, and above such temperatures (about 25° C), *L. obtusata* is a "nonactive endurer." In *Littorina littorea,* aerial and aquatic respiration are almost exactly equal up to 30° C (corresponding to normal upper limits for their environment). Unlike *Acmaea* and *L. obtusata,* increase in uptake between 15° C and 30° C is considerably less than between 5° C and 15° C (or, for that matter, above 30° C), being a zone of some metabolic regulation, in both aerial and aquatic respiration. Finally, *Littorina saxatilis* shows somewhat lower aerial than aquatic respiration rates at all temperatures. Decreasing rates above 25° C are still well below thermal deterioration, and this represents a reversible "torpor" or short-term diapause, which fits with the specific habitat (70% to 90% of their time out of water). It is worth noting that these major shifts, with changed temperature and between aerial and aquatic respiration, are many times greater than the rhythmic metabolic shifts known to occur under near-constant conditions.

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Reduction of radiation-induced mitotic delay in sea urchin eggs following prefertilization ionic stimulation. Ronald C. Rustad, Elaine I. Cassen, and Soheyla D. Gharib.

Exposure of unfertilized sea urchin eggs to ammoniated sea water is known to increase the K^+ conductance and the rate of protein synthesis and to "trigger" DNA synthesis and chromosome condensation. We have shown previously that prefertilization ammonia treatments can reduce the duration of the first cleavage cycle. In addition, prefertilization exposure to ammonia led to a reduction in the amount of mitotic delay which ordinarily arises from γ-irradiating the sperm, unfertilized eggs, or zygotes. Normally, the sensitivity to γ-radiation-induced mitotic delay increases continuously for about 15 minutes after fertilization (apparently because progressively less time is left for recovery prior to a radiation-sensitive mitotic event). Thereafter, the sensitivity decreases to zero in two distinct steps which we have proposed represent the times at which two separate radiation-sensitive mitotic events occur.

Eggs of the sea urchin *Arbacia punctulata* were exposed to ammoniated sea water and fertilized. Different samples of the fertilized eggs were irradiated at intervals thereafter. In some experiments, there was no time-dependent increase in radiation sensitivity in the ammonia-treated eggs, while the unstimulated eggs exhibited the usual increase. The uniform response could occur if the damage leading to the delay of the first radiation-sensitive mitotic event (but not the second) were reparable during the first 15 minutes after fertilization, and ammonia stimulated the egg to complete that mitotic event before irradiation. In other experiments, the ammonia-treated, irradiated eggs divided before the control eggs. Thus, all of the radiation-sensitive mitotic events appeared to have been completed within 5 minutes after fertilization and possibly even prior to sperm entry.

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Conversion of D-glucose to L-ascorbic acid in Cyclotella cryptica *Reimann, Lewin and Guillard.* James A. Saunders, R. R. L. Guillard and Frank A. Loewus.

Cyclotella cryptica Reimann, Lewin and Guillard is a brackish-water centric diatom capable of growth in the dark on D-glucose after a period of dark adaptation. This observation provides basis for the present study, conversion of D-glucose to L-ascorbic acid. Cells which had been grown for periods ranging from 10 to 21 days on f/2 media were held in the dark for 48 hours and then given trace amounts of specifically labeled D-glucose. After an additional 48 hours in the dark, cells were harvested by centrifugation, washed and ground in 0.1% oxalic acid. Labeled ascorbic acid was trapped with carrier and separated from other acidic constituents by ion exchange column procedures. When D-gluocse-1-^{14}C was provided 33 to 50% of the label was taken up and over 1% of it appeared in ascorbic acid. Two recrystallizations from glacial acetic acid brought this ascorbic acid to constant specific radioactivity. Preliminary studies on distribution of label revealed extensive fragmentation of D-glucose preceeding ascorbic acid synthesis. Experiments with D-glucose-6-^{14}C, -1-^{3}H and -6-^{3}H supported this observation on the redistribution of label. In conclusion, *C. cryptica* is able to utilize D-glucose for L-ascorbic acid biosynthesis in the dark but the process appears to be unlike that involving D-glucose utilization in higher plants.

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Long-term (3 to 5 year) records of gene frequencies in natural populations of an abundant, sub-tidal species (the bryozoan Schizoporella errata*).* THOMAS J. M. SCHOPF.

The relative frequency of occurrence between the two alleles of a locus for leucine amino peptidase appears adjusted to local ecologic conditions, especially the warmest summer temperatures. (1) The cline (0.50 to 0.94) in allele frequencies over the 100 km distance from Cuttyhunk (western end Elizabeth Islands) to the eastern end of Cape Cod (Orleans) reflects a temperature cline (7° C) in summer temperatures. This pattern is reflected in population samples from 15 localities in 1974 data, repeating a similar but more pronounced cline in 1972 and 1973. (2) For the past 3–5 years, we have been in a warming trend in water temperatures offshore. This is reflected in a significant increase in the "warm-water" allele in my offshore, cooler localities: Cuttyhunk Channel, Robinsons Hole, and off Woods Hole. The "warm-water" allele increased by 0.16 to 0.19, depending upon the place, over the 5 year period. (3) Temperature in coastal areas is chiefly a function of local hydrography due to patterns of sediment movement in storms. These areas are normally much warmer than offshore areas. However, at one locality (New Seabury), the warm-water allele decreased significantly in frequency (by 0.15) over a 3 year period. This has paralleled changes in circulation which have caused a more direct exchange with the open sea. (4) At most harbor localities, no significant change in allele frequencies was noted thus reflecting more uniform conditions in protected areas. (5) For at least this marine species, natural populations appear to be evolving in response to local ecologic conditions over very short periods of time.

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Activation of immature starfish oocytes by a divalent ionophore and sperm. ALLEN W. SCHUETZ.

Previous studies indicate that divalent ions are involved in mediating activation of mature oocytes. *In vitro* effects of the divalent ionophore (A23187) on starfish (*Asterias forbesi*) oocytes at various stages of meiotic maturation were examined in order to investigate onset of activation. Immature (germinal vesicle stage) oocytes were collected from ovaries, preincubated in calcium free sea water, by artificial spawning in filtered sea water. Germinal vesicle breakdown and reinitiation of meiosis were induced by adding 1-methyladenine to ovaries or isolated immature oocytes. Oocytes were rapidly activated (vitelline membrane elevation) by A23187 following germinal vesicle breakdown and throughout the period of first and second polar body formation. Meiosis proceeded normally in the continual presence of ionophore. In contrast to its effects on *Spisula* oocytes, ionophore addition to immature starfish oocytes did not initiate maturation, but did not initiate a rapid activation response. Prematurational activation of oocytes was also obtained following gamete exposure to starfish sperm. Addition of *Arbacia, Echinarachnius, Spisula* or *Hydroides* sperm to starfish oocytes in either the pre or post maturation stage did not induce activation. Immature oocytes activated by sperm or ionophore underwent maturation following 1-methyladenine treatment but did not undergo normal embryonic development. Sperm or ionophore activated immature oocytes, fertilized following 1-methyladenine induced maturation, also did not undergo embryogenesis. Fertilized, 1-methyladenine matured oocytes underwent embryogenesis. Results demonstrate that the oocyte activation response can be initiated independent of nuclear maturation process and suggest that hormone induced cytoplasmic factors may not be involved in mediating activation. Furthermore, the oocyte meiotic stage at the time of fertilization is crucial in determining the incidence of subsequent development.

Quantification of light produced from hydrozoan photocytes. JEANNETTE M. SHOREY AND JAMES G. MORIN.

We know of no published data concerning the amount of light emitted from individual light-producing cells in any multicellular animal. We report here such information, though preliminary, for the colonial hydroids, *Clytia edwardsi* and *Obelia geniculata.*

It has been demonstrated at previous MBL General Meetings that the luminescent cells of hydroids and alcyonarians can be visualized by fluorescence microscopy as bright green photocytes. Using this technique, photocytes within individual uprights and adjacent stolons of each hydroid were quickly counted. Each piece of counted tissue was transferred to a scintillation vial, containing 1 ml sea water. One ml isosmolar KCl was injected into the vial which had been placed over a photomultiplier. The integral of the total light emitted was recorded on a polygraph. Additional stimulation by KCl did not elicit greater light output, therefore we assume our data represent the total emission capacity of photocytes at any given time. Results were calibrated against a known light standard.

It was anticipated that a linear relationship should exist between the number of photocytes in a given tissue and its total light emitted. Our initial results confirmed this assumption. Therefore, using the slopes derived from a statistically significant linear regression of our data, we determined the photocyte output to be about $1\text{–}2 \times 10^8$ quanta/cell, with a range of extreme values of $0.2 - 6 \times 10^8$ quanta/cell, for both *Clytia* and *Obelia.* With these techniques, we found no statistically significant differences in light output from photocytes variously localized within uprights and stolons.

The reported values probably represent the lower limit of light emitted. This lower limit and the variability observed can be explained from the following: a) internal absorption by tissues, perisarc, and epiphytes, b) non-random light emission, c) biological rhythms, d) genetic variations among the sampled populations, e) nutritional and environmental differences, f) age of upright tissues, and g) prior stimulation during the various manipulations.

Isolation of spore-forming microorganisms from Little Sippewissett marsh. HOWARD J. SINGER AND E. R. LEADBETTER.

The presence and relative numbers of aerobic spore-forming bacteria in different portions of this salt marsh were determined by quantitative dilution and plating on peptone-sea water (Pep-SW) and peptone-distilled water (Pep-DW) agar-solidified media before and after pasteurization (80° C, 10 min) of samples from sandy and mud-bottom ponds, running water, and submerged algal mats.

Species of *Bacillus* were categorized initially as: (a) isolates unable to grow in the absence of Pep-SW, (b) isolates able to grow in Pep-DW, but with faster growth rates in Pep-SW, or (c) isolates able to grow in Pep-DW as well as or better than in Pep-SW. The one type "a" isolate examined was unable to grow in Pep-DW containing up to 3% (w/v) NaCl, although it would grow in Pep-DW containing 20% (v/v) (the lowest concentration tested) sea-water or MBL Standard Artificial Seawater. No growth was detected on Pep-DW-agar when up to 10^7 viable cells were plated.

Solidified media containing 80% (v/v) SW, 1% (w/v) NH_4Cl, and 1% (w/v) of either acetate, succinate, sucrose, starch, casamino acids, or glucose were employed in determining the ratio of spore-forming and non-spore-forming bacteria present in the samples and able to grow on the given medium. Less than 1% of the cultivable aerobic bacteria were spore-formers. Highest viable counts were obtained with casamino acids or succinate, while glucose and acetate were poorest in this regard. Of twelve isolates selected for further study, eight formed red, yellow, or orange colonies after prolonged incubation for four or more days at 25° C. The isolates were not readily equatable with well-recognized *Bacillus* species.

Studies on protein metabolism and growth in fish. MICHAEL A. K. SMITH AND A. E. V. HASCHEMEYER.

Toadfish, mean weight 300 g, kept in running seawater at ambient summer temperature 22° C and fed killifish *ad libitum,* show a specific growth rate of about 0.4%/day. Studies were begun to assess protein synthetic activity in various tissues in relation to protein accumulation. Incorporation of ^{14}C-leucine into liver protein assayed at 6 hrs was similar for both hepatic portal vein and gill artery injection and for tracer or 100 mM leucine concentrations. Incorporation into gill protein per unit tissue weight was comparable to that of liver but that in muscle was only about 1/30th as much. Distribution of protein radioactivity as % of dose per total tissue at $t = 6$ hr was as follows: liver 6.3%; muscle 5.4%; gill 1.8%. Free radioactivity in the tissues at this time was: liver 1.2%. Proportion of total body weight contributed by these tissues is: liver 2.5%; muscle 60%; gill 0.7%. Measurements of free leucine concentration in the various tissues by automatic analysis together with total (protein + free) radioactivities yielded estimates of leucine pool specific activities. These values combined with the protein incorporation data yield ratios of protein synthesis in muscle and gill (per 100 g body weight), relative to liver, of about 4.5 and 0.3, respectively. If it is assumed that the time course of utilization of ^{14}C-leucine is similar in the three tissues, then true protein synthetic rate in muscle and gill may be calculated from the known synthesis rate in liver of 22°-acclimated toadfish (42 mg protein/100 g body wt/day) obtained in rapid pulse experiments. The results are 190 and 13 mg/100 g/day for muscle and gill respectively. Comparison with growth rate data indicates that the per cent of newly synthesized protein accumulated under these conditions is about 3% for liver and gill and 20% for muscle.

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The life-cycle of the gasterostome trematodes, Rhipidocotyle transversale *Chandler, 1935 and* Rhipidocotyle lintoni *Hopkins, 1954.* HORACE W. STUNKARD.

McCrady (1874) described a gasterostome cercaria from the American oyster, *Ostrea virginica* taken at Charleston, South Carolina, as a new species, *Bucephalus cuculus.* Linton (1905) described metacercariae encysted in *Menidia menidia* and adult worms from the gar, *Tylosurus* (= *Strongylura marinus*) taken at Beaufort, North Carolina, as *Gasterostomum gracilescens* (Rudolphi, 1819). Tennent (1905, 1906, 1909) found the parasite of the oyster and reported the life-cycle. He postulated that the parasite from the American oyster is identical with the one from the European oyster described by Lacaze-Duthiers (1854), that it is identical with the metacercaria in *M. menidia,* and that it is the larva of *G. gracilescens.* None of these postulates is correct. No life-cycle of an American marine gasterostome has previously been discovered. Chandler (1935) described encysted gasterostome metacercariae from *M. menidia* in Texas as *Rhipidocotyle transversale* n. sp. Hopkins (1954) noted that the metacercariae in *M. menidia* are identical with the adults in the gar and the species was identified as an *R. transversale.* Hopkins described three gasterostome species from *S. marinus.* One was identified as *R. transversale,* one was described as a new species, *Rhipidocotyle lintoni,* and the third as a new species, *Bucephaloides strongylurae.*

Juveniles of both *R. transversale* and *R. lintoni* are common in *M. menidia* at Woods Hole. Both species may occur in the same fish and the intensity of infection varies from one to many worms; usually the number is two to four. They are encysted in muscles throughout the body and have been taken from the surface of the brain. *Menidia menidia* and *S. marinus* occur from Cape Cod to Texas and since gasterostomes utilize bivalve mollusks as first intermediate hosts, some species with the same distribution and from the same ecological milieu must be involved in the life-cycle. The number of species that meet these requirements is not large and the host of the parasite has been discovered. It is *Lyonsia hyalina.* Developmental stages of the gasterostomes are under study.

Energy transfer between fluorescent probe molecules in and across nerve membrane. I. TASAKI, A. WARASHINA AND H. PANT.

Resonance transfer of energy was studied by incorporating two kinds of fluorescent probes into the membrane of crab nerve and squid giant axons. A solvent-polarity sensitive probe, p-C1-2, 6-anilnonaphthalene-sulfonate, was used as the donor of energy; merocyanine-540 was employed as the acceptor. These probes are known to produce fluorescence signals in response to electric stimulation. Physico-chemical properties of these probes were examined *in vitro* as well as after incorporation into the nerve membrane. The transition moments of these molecules in the membrane were found to be oriented predominantly in the direction normal to the surface. The emission spectrum of the donor (410–570 nm) was shown to overlap the absorption spectrum of the acceptor. The absorption spectrum of the acceptor was complex indicating the existence of both strongly fluorescent monomers and weakly fluorescent dimers (or aggregates) in the nerve membrane. Measurements of absorption changes associated with action potentials showed that there is an increase in the number of monomer molecules (with an absorption maximum at about 580 nm) accompanied by a simultaneous decrease in dimers. The nerve membrane stained the acceptor produced extremely weak fluorescence when optically excited at 365 nm (roughly corresponding to an absorption minimum). Addition of the donor to the nerve membrane brought about a distinct enhancement of the acceptor emission. This enhancement was observed when the donor and acceptor molecules were placed on the opposite side as well as on the same side of the membrane. The observed phenomena are interpreted on the basis of Förster's theory of resonance energy transfer.

In vivo *contractility of amoeba cytoplasm.* DOUGLASS L. TAYLOR.

The rheological and contractile properties of ectoplasm and endoplasm were studied in single specimens of *Chaos carolinensis.* Cytoplasmic viscoelasticity was demonstrated by applying quick stretches to microneedles inserted into various areas of the cell. The ectoplasm in both the tail and the advancing pseudopods were highly viscoelastic without any obvious gradient. In contrast, the endoplasm just behind the fountain zone was more viscoelastic than uroid endoplasm.

Physiological buffers were micro-injected into intact cells in order to compare the chemical control induced previously in isolated cytoplasm with the effects on endoplasm and ectoplasm *in vivo.* Small volumes (ca. 0.0001 μl) of a contraction solution containing the threshold calcium ion concentration caused local contraction of the endoplasm and ectoplasm at the site of injection in both the uroid and advancing pseudopods. However, larger volumes of this solution or higher calcium ion concentration caused large contractions of endoplasm and ectoplasm at the site of injection. Micro-injection of this solution into endoplasm just behind the fountain zone caused contraction of the ectoplasm and endoplasm which reversed the direction of cytoplasmic streaming. Micro-injection into the uroid increased the rate of streaming out of the uroid while producing a hyaline cap at the site of injection.

Micro-injection of a relaxation solution into the uroids of advancing cells caused the local loss of ectoplasmic structure. Streaming continued out of the uroids until more than half the ectoplasm lost its visible structure. Relaxation solution induced the reversal of cytoplasmic streaming when injected into advancing pseudopods. These effects were completely reversible.

These results indicate that here is an increasing gradient of endoplasmic viscoelasticity from the uroid to the tips of advancing pseudopods. In addition, endoplasm and ectoplasm are contractile at all points in the cell so that the site of calcium release or influx will identify the normal site of contraction. In addition, an efficient calcium sequestering mechanism must be present in the cell.

Induction of polyspermic fertilization of Arbacia *eggs by specific protease inhibitors leupeptin and antipain.* WALTER TROLL, HERBERT SCHUEL, AND WALTER L. WILSON.

Cortical granules in sea urchin eggs contain a trypsin-like protease which is secreted into the ambient sea water during fertilization. Previous studies with soybean trypsin inhibitor suggested that the protease functions in the discharge of the cortical granules, elevation of the fertilization membrane, and establishment of the block to polyspermy. Leupeptin and antipain are small peptide aldehydes isolated from the fermentation product of actinomycetes by Umezawa and colleagues which are potent competitive inhibitors of proteolytic enzymes. *Arbacia* eggs fertilized in the presence of leupeptin and antipain (5×10^{-5} to 10^{-3} M) do not elevate normal fertilization membranes and became so heavily polyspermic that first cleavage was delayed or prevented entirely. Eggs were also initially inseminated in the presence of 5×10^{-4} M antipain and leupeptin with a low concentration of sperm just sufficient to produce simultaneous monospermic fertilization of all the eggs, and were then re-inseminated with a high concentration of sperm at later times. These experiments showed that polyspermy resulted from a process of re-fertilization extending for a period of about 10 to 15 min post initial fertilization. Normal cleavage and development was obtained when eggs were placed in 10^{-3} M leupeptin and antipain after the fertilization membrane had elevated. These data show that the cortical granule protease is inhibited by leupeptin and antipain, and confirm the critical secretory function of this protease in establishing the block to polyspermy in sea urchin eggs.

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Distribution of adenosine-5′-phosphosulfate (APS) and adenosine-3′-phosphate-5′-phosphosulfate (PAPS) sulfotransferases in assimilatory sulfate reducers. MONICA LIK-SHING TSANG AND JEROME A. SCHIFF.

A distinction between dissimilatory sulfate reducers which utilize sulfate as an electron acceptor in respiration and assimilatory sulfate reducers which use sulfate to satisfy their requirements for reduced sulfur compounds for growth is based on the type of activated sulfate compound used as the immediate donor for reduction. Dissimilatory sulfate reducers have been shown to utilize APS and it was suggested that assimilatory sulfate reducers use PAPS. With our findings that among assimilatory sulfate reducers, *Chlorella pyrenoidosa* uses APS as the substrate for reduction via APS-sulfotransferase while *Escherichia coli* uses PAPS via a PAPS-sulfotransferase, this correlation does not appear to hold, and we have undertaken a phylogenetic survey of APS and PAPS utilization by reducing systems to determine the significance of their distribution. We find that non-photosynthetic organisms such as *E. coli* among the procaryotes and yeast among the eucaryotes contain PAPS-sulfotransferases while organisms which are photosynthetic such as the red algae, *Chondrus crispus* and *Porphyridium aerugineum,* the brown alga, *Fucus vesiculosus,* the diatoms *Cyclotella* and *Chaetocerus pseudocrinitis,* the dinoflagellates *Peridinium trochoidium* and *Gymnodinium* sp., the cryptophyte *Chroomonas* sp., the prasinophyte *Platymonas* sp., and the blue-green algae *Oscillatoria wononichinii* and *Synechococcus* sp. as well as spinach chloroplasts all utilize APS rather than PAPS. This suggests that APS utilization for assimilatory reduction is characteristic of chloroplasts and chloroplast-containing organisms while PAPS utilization for assimilatory reduction is characteristic of non-photosynthetic organisms. It is possible that the primitive case, a relict of the anaerobic phase of life, is represented by the dissimilatory sulfate reducers. Assimilatory sulfate reduction may have evolved from this point, when oxygen entered the atmosphere, in two paths: the aerobic bacteria utilizing PAPS leading to other non-photosynthetic organisms and the blue-green algae utilizing APS leading to chloroplasts and chloroplast-containing organisms.

We thank Dr. Robert Guillard and Dr. Joe Ramus for supplying many of the organisms used.

Skin photoreceptors in the leopard frog. GEORGE WALD AND STEPHEN RAYPORT.

In addition to old reports of behavioral responses (orientations, motions) excited by light striking the skin in frogs and tadpoles (G. H. Parker, *Am. J. Physiol.,* **10**, 28, 1903; V. Obreshkove, *J. Exp. Zool.,* **34**, 235, 1921), there have been recent reports of both fast photovoltages and slow electrophysiological responses from isolated pieces of frog skin (Becker and Cone, *Science*, **154**, 1051, 1966; Becker and Goldsmith, *Nature*, **220**, 1236, 1968). We, like the latter workers, have clamped pieces of skin in a modified Ussing cell, bathed on both sides with frog Ringer, with light-shielded Ag-AgCl electrodes dipping into the Ringer. We have been concerned only with the slow response (electrodermogram, EDG). It has a latency at 23° C of about 0.75 sec takes another second to peak, and about 2 sec more to return to the base-line. The skin has a resting potential in the dark of up to 100 mv, outer surface negative; and this is increased (hyperpolarized) by light by as much as 0.8 mv. Over our working range the amplitude of the EDG is proportional to the light intensity. The spectral sensitivity of photoexcitation ranges between 320 and 470 nm, and is maximal at about 395 nm. Repeated flashes in the near ultraviolet cause a large decrease in response (adaptation) that shows no recovery within 70 min in the dark at 23° C. Exposures to longer wavelengths, however, cause a strong photorecovery. The photorecovery spectrum overlaps with the photostimulation spectrum in the violet, but extends to about 560 nm, peaking near 500 nm. These observations indicate a photoreversible system involving two stable states, photostimulatory peaking at about 395 nm, and photorecovery peaking near 500 nm.

Supported by funds from NIH, NSF and Harvard University.

A general method for the introduction of enzymes, by means of liposomes, into lysosomes of deficient cells. GERALD WEISSMANN, DAVID BLOOMGARDEN, ROBERTA KAPLAN, CHARLES COHEN, SYLVIA HOFFSTEIN, TUCKER COLLINS, AVRUM GOTTLIEB AND DAVID NAGLE.

The phagocytes of the smooth dogfish (*Mustelus canis*) contain no endogenous peroxidase within their lysosomes and therefore can be used as models for cells genetically deficient in lysosomal enzymes such as myeloperoxidase. Based upon our previous work showing (1) the possibility of trapping enzymes in the aqueous interspaces between the lipid layers of liposomes, and (2) the possibility of coating liposomes with immunoglobulins, we have managed to obtain incorporation of over 50% of exogenous horse-radish peroxidase by dogfish phagocytes, provided the enzyme is exhibited to cells after incorporation into liposomes (prepared with ovolecithin, dicetylphosphate and cholesterol in molar ratios of 7:2:1) and coating these with heat-aggregated IgM (62°, 10 min). Trapping of peroxidase by liposomes was established by chromatography of *free* and *liposome-bound* enzyme and markers of the aqueous space (glucose, chromate anion) on Sephadex G-200, Sepharose 2B and Sepharose 4B: 0.67 (mean) micromoles of markers and 313 nanograms of peroxidase were trapped per micromole of phospholipid $(n=9)$. Enzyme and marker trapping were not electrostatic, varied with the molar ratio of charged component (dicetylphosphate), and was reversed after disruption of liposomes by Triton X-100 which showed these to be 98–100% "latent" within liposomes. Uptake $(n=12)$ of dogfish aggregated IgM-coated liposomes at 30° (9.7 ng/10^6 cells/hr) exceeded that of free enzyme (less than 1 ng), of uncoated liposomes (4.1 ng), of native IgM-coated liposomes (4.8 ng), of aggregated IgM-coated liposomes at 4° (5.7 ng), and of these treated with cytochalasin B to block phagocytosis (5.5 ng) or cytochalasin B-treated cells held at 4° (2.0 ng). Once taken up by phagocytes, over 50% of enzyme was latent within cells (as determined by lysis in distilled water) and sequestered exclusively within lysosomes (localized by ultrastructural histochemistry). This is a general method for the provision of exogenous enzymes to phagocytic cells genetically deficient in lysosomal hydrolases.

Evidence for biased bipolar polymerization of actin filaments. D. T. WOODRUM, S. RICH AND T. D. POLLARD.

Actin filaments polymerize in a biased bipolar manner judging from experiments where actin monomers are polymerized onto the ends of preformed actin filaments decorated with heavy meromyosin (HMM). Purified rabbit skeletal muscle actin was depolymerized by dialysis against 2 mM tris, pH 7.8, 0.1 mM $CaCl_2$, 0.2 mM ATP to yield monomeric G-ATP-actin. This monomeric actin was then passed through a Sephadex G-25 column equilibrated with 2 mM tris, pH 7.8, 0.1 mM $CaCl_2$, 1 mM DTT to remove unbound ATP from the solution of G-ATP-actin. Samples of actin were polymerized by the addition of KCl to 0.1 M and then decorated with a slightly substoichiometric concentration of HMM for use as nuclei in the following experiments.

Addition of G-ATP-actin to the nuclei resulted in the growth of bare segments of actin from the ends of the decorated nuclei. The extent and the polarity of this nucleated polymerization was assessed by electron microscopy of negatively stained specimens. When low concentrations of monomers (0.1 mg/ml or less) were added to nuclei, newly polymerized actin was found at the "barbed" end of 85% of the nuclei, while none of the nuclei had bare additions at the pointed end. At higher concentrations of monomer (0.5 mg/ml), bare additions were observed at *both* ends of the nuclei, although the additions were 4–8 times longer at the barbed end compared with the pointed end. Variation of the concentration of nuclei (0.05–0.5 mg/ml), the temperature (0–25° C) and the presence or absence of 1 mM Mg^{++} had no influence on the direction of nucleated actin polymerization.

How cell growth is inhibited by near UV light photoproducts of tryptophan. S. Zigman, T. Yulo, G. Griess, B. Antonellis, and R. Rustad.

Exposure of neutral pH, aqueous solutions of tryptophan (TRP) to near UV light (320 to 400 nm), termed NUVTRP, produces photoproducts with growth inhibitory properties. We report here that the growth of mouse embryonic fibroblasts (MEF), dogfish (*Mustelus canis*) eye lens epithelial cells and sea urchin (*Arbacia punctulata*) fertilized eggs is also strongly inhibited by dilute solutions of TRP (5×10^{-4} M) which were previously exposed to 3 mW/cm^2 of near UV light from several to 24 hrs.

For MEF cells in Eagle's MEM, growth and synthesis of protein, RNA, and DNA were markedly inhibited (70 to 90%) by long term (3-day) exposure to NUVTRP. One-hr exposures to NUVTRP were also effective in appreciably inhibiting precursor incorporation into protein, RNA, and DNA. Gel filtration (Sephadex G 10) of whole NUVTRP yielded a yellow brown fraction (400 dalton Mol. Wt.) which had the greatest antigrowth activity. This was not N-formylkynurenine, a known photoproduct of TRP.

The incorporation of amino acids, uridine and thymidine into eye lens epithelial cell protein, RNA, and DNA was also markedly inhibited when NUVTRP was added at 5×10^{-4} M to the elasmobranch Ringer's medium used for the incubations.

For sea urchin eggs, Millipore filtered sea water plus TRP at the same level prevented structuralization of the mitotic apparatus prior to the first cleavage, approximately doubled division time, and led to abnormal multicelled stages. During the first hr postfertilization, protein, RNA, and DNA incorporated ^{14}C and ^{3}H-amino acids, ^{3}H-uridine, and ^{3}H-thymidine at less than half the rate as the TRP controls. Exposure of sea water with the same level of TRP to window-glass-filtered sunlight for 8 hrs produced similar growth inhibition. The photoproduct fraction most active against the growth of MEF cells was also found most active in the sea urchin system.

NUVTRP inhibits the growth of these cells by interfering with macromolecular synthesis. Abnormal mitotic apparatus formation may relate to deficient macromolecule supply. Interference with polymerases and energy supplying enzymes may also be involved.

Supported by PHS grant EY-00459; Monroe Cty. Cancer and Leukemia Society; ONR-CNA Univ. of Rochester Contract; U.S.A.E.C.

Studies on the establishment of a cytochalasin B sensitive rapid polyspermy block following fertilization of Spisula solidissima *eggs.* Carol Ann Ziomek and David Epel.

The block to polyspermy has been best studied in the sea urchin eggs which appear to have two polyspermy blocks, a fast partial block and a slower complete block resulting from the cortical reaction. The eggs of *Spisula solidissima* also have a polyspermy block but no cortical reaction. Thus, a study of this organism may provide insight into the general blocks not involving the cortical granules.

The timing of the block in *Spisula* eggs was determined using the polyspermy assay of Rothschild and Swann. One culture was lightly inseminated resulting in 100% monospermy. A second culture was heavily inseminated leading to 100% polyspermy. A third series of cultures were lightly inseminated and followed at various times by a heavy sperm dose. In *Spisula* eggs, the half-time for completion of the block was 2.5 sec.

The vitelline layer was removed from unfertilized *Spisula* eggs by a 2 min incubation with 1 M β-alanine containing 5 mM EDTA, pH 6.5, followed by washing two times with filtered seawater. The timing and extent of the block to polyspermy was identical to untreated eggs. Therefore it appears that the block to polyspermy involves changes in the plasma membrane.

Spisula eggs were then pretreated for 10 min with various concentrations of cytochalasin B (0.5, 1.0, and 5.0 μg/ml), washed and subjected to the polyspermy assay. In all cases, the eggs became polyspermic upon addition of heavy sperm doses. If the cytochalasin B was added after the block was established and then heavy sperm doses added, no polyspermy was detected. Therefore it appears that the establishment of the block is sensitive to cytochalasin B but insensitive once the block has been established.

This study was performed in the Fertilization and Gamete Physiology Research Training Program at the Marine Biological Laboratory (NIH Grant 3-T01-HD00026-12S1).

Continued from Cover Two

4. ***Literature Cited.*** The list of references should be headed LITERATURE CITED, should conform in punctuation and arrangement to the style of recent issues of THE BIOLOGICAL BULLETIN, and must be typed *double-spaced* on separate pages. Note that citations should include complete titles and inclusive pagination. Journal abbreviations should normally follow those of the U. S. A. Standards Institute (USASI), as adopted by BIOLOGICAL ABSTRACTS and CHEMICAL ABSTRACTS, with the minor differences set out below. The most generally useful list of biological journal titles is that published each year by BIOLOGICAL ABSTRACTS of those abstracted (most recent issue: November, 1972). Foreign authors, and others who are accustomed to use THE WORLD LIST OF SCIENTIFIC PERIODICALS, may find a booklet published by the Biological Council of the U.K. (obtainable from the Institute of Biology, 41 Queen's Gate, London, S.W.7, England, U.K. at £0.65 or $1.75) useful, since it sets out the WORLD LIST abbreviations for most biological journals with notes of the USASI abbreviations where these differ. CHEMICAL ABSTRACTS publishes quarterly supplements of additional abbreviations. The following points of reference style for THE BIOLOGICAL BULLETIN differ from USASI (or modified WORLD LIST) usage:

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Volume 147 Number 3

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THE BIOLOGICAL BULLETIN accepts original research reports of intermediate length on a variety of subjects of biological interest. In general, these papers are either of particular interest to workers at the Marine Biological Laboratory, or of outstanding general significance to a large number of biologists throughout the world. Normally, review papers (except those written at the specific invitation of the Editorial Board), very short papers (less than five printed pages), preliminary notes, and papers which describe only a new technique or method without presenting substantial quantities of data resulting from the use of the new method cannot be accepted for publication. A paper will usually appear within four months of the date of its acceptance.

The Editorial Board requests that manuscripts conform to the requirements set below; those manuscripts which do not conform will be returned to authors for correction before review by the Board.

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2. *Tables, Foot-Notes, Figure Legends, etc.* Tables should be typed on separate sheets and placed in correct sequence in the text. Because of the high cost of setting such material in type, authors are earnestly requested to limit tabular material as much as possible. Similarly, foot-notes to tables should be avoided wherever possible. If they are essential, they should be indicated by asterisks, daggers, etc., rather than by numbers. Foot-notes are not normally permitted in the body of the text. Such material should be incorporated into the text where appropriate. Explanations of figures should be typed double-spaced and placed on separate sheets at the end of the paper.

3. *A condensed title* or running head of no more than 35 letters and spaces should be included.

Continued on Cover Three

Vol. 147, No. 3 December, 1974

THE
BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

ULTRASTRUCTURE OF A CEPHALOPOD PHOTOPHORE. I. STRUCTURE OF THE PHOTOGENIC TISSUE

Reference: *Biol. Bull.*, **147**: 507–521. (December, 1974)

JOHN M. ARNOLD AND RICHARD E. YOUNG

Pacific Biomedical Research Center, University of Hawaii, Honolulu, Hawaii;
Marine Biological Laboratory, Woods Hole, Massachusetts; and Department
of Oceanography, University of Hawaii, Honolulu, Hawaii

Of the many animal taxa which are capable of light production, the Cephalopoda present some of the most varied and elaborate photophores known. Photogenic organs are found within the mantle (Clarke 1965) in or by internal organs (*e.g.*, ink sac; Boletzky, 1970) or on the siphon (Hoyle, 1904), on the skin of the mantle, head and/or tentacles, and on the surface of the eye (Berry, 1920a, 1920b). There are many different types of photogenic organs and several dif-

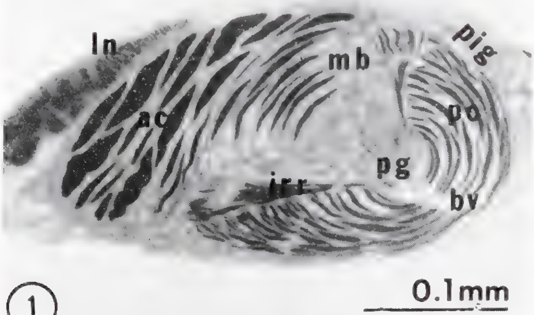

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FIGURE 1. Light micrograph of a whole photophogenic tissue (pg) composed of an axial cone and spheroidal knob enclosed in a posterior cup (pc) made of regular iridophores. The anterior cap (ac) grades into a conical "plug" of iridophores in the center of the axial cone. Distal to the anterior cap is the clear lens (ln) composed of iridophores with many iridosomes in each cell. Surrounding the axial cone is a conical layer of irregular iridophores (irr). The posterior cup and proximal side of the whole organ is covered with a layer of brownish pigment (pig). Muscle bands (mb) and blood vessels (bv) occasionally penetrate the posterior cup or irregular iridophores.

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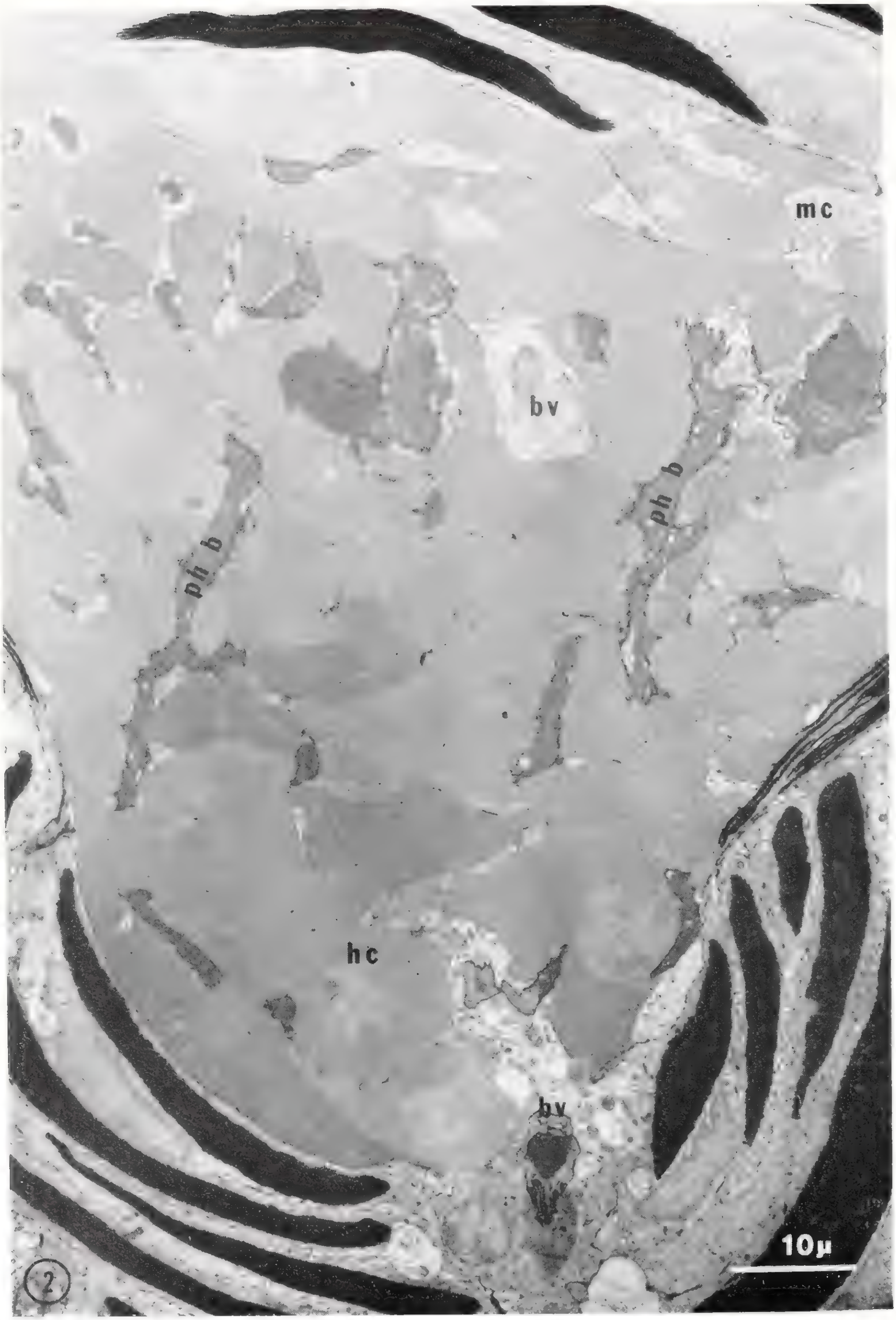
mc
bv
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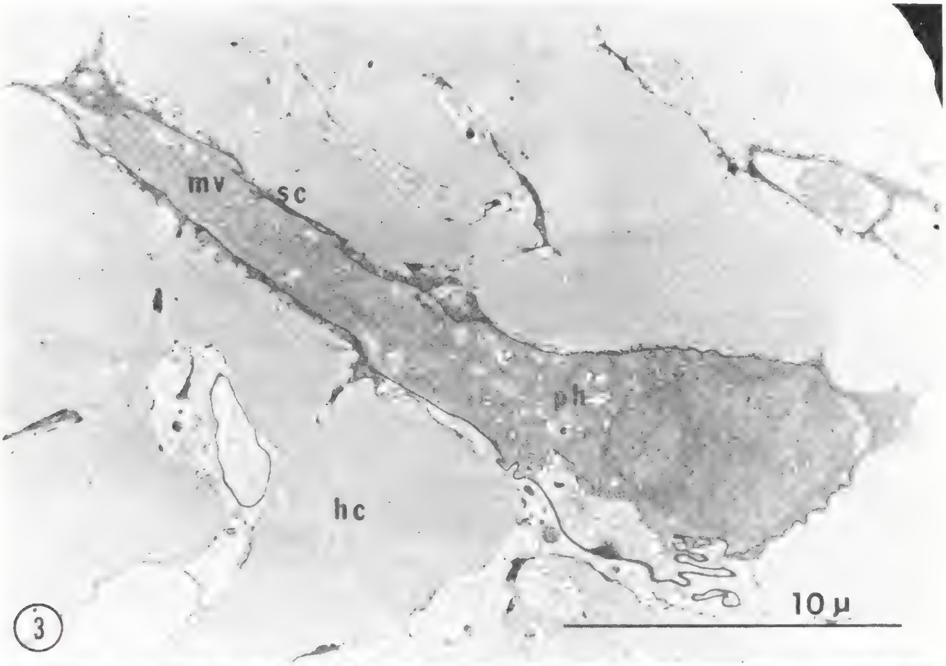
ph b
hc
bv
10μ
2

ferent types are often present simultaneously in the same individual and even on the same organ. The function of the photogenic organs is largely unknown, but it has been suggested that they are important in countershading (Young, 1972, 1973; Clarke, 1963), predation (Nicol, 1960), and possibly sexual interaction (Clarke, 1963, Young, 1974) or schooling behavior. Both bacterial and autophotogenic organs have been reported in cephalopods (Nicol, 1960). The autophotogenic photophore presents an interesting opportunity to study the production of light by a tissue whose function is the opposite of the retina but which still has many basic similarities to photoreceptive organs. By a careful fine structural analysis of these photogenic organs, insight might be gained into the basic structural mechanism of interconversion of chemical and electrical energy into light which, in turn, might be compared in interesting ways to photoreception. The purpose of these two papers is to report the results of ultrastructual studies on one common type of photogenic organ in the squid *Pterygioteuthis microlampas,* a midwater, Pacific species which probably only encounters low levels of light, and to speculate on the possible function of these photophores.

Although Hoyle (1904) has described the histological structure of a photophore of a closely related squid (*Pterygioteuthis giardi*), for purposes of convenience to the reader and because there are some slight differences in our observations, the following brief description of the whole photophore is provided. There are several photophores located on the ventral surface of each eye, and these are of at least three types. Eleven of these photophores have similar structure and have been the subject of this study. These photophores vary in size, depending on the individual squid, but typical ones measure about 0.4 mm × 0.3 mm, are lentoid in shape, and are roughly radially symmetrical. Hoyle (1904) described a connective tissue cap which was reddish-brown; a "posterior cup" of oval or circular fibrous "scales" (=iridophores) in concentric layers; an "anterior cap" of "scales"; an "inner funnel" of rather coarse fibers (=irregular iridophores); and a central mass filled with almost structureless parenchymous tissue which is composed of a "spheroidal knob" and an "axial cone" (Fig. 1). In this paper this tissue will be referred to as the photogenic tissue and the presumed light producing cell will be called the photocyte. In addition there is also a clear layer of modified iridophores (=lens) distal to the anterior cap, which Hoyle did not include in his description. Nerves and blood vessels occasionally penetrate the sides or the base of the bulb region of the posterior capsule. Bands of muscle traverse the "inner funnel" region and attach to those iridophores which make a conical proximal "plug" as a central continuation of the anterior capsule. Apparently, each of the three types of iridophores making up the posterior and anterior capsules, the "inner funnel" and clear "lens" have different functions, and this paper will be devoted to a description of their ultrastructure and speculation as to their functions.

FIGURE 2. Photogenic tissue of the spheroidal knob and part of the axial cone. A blood vessel (bv) enters through the posterior cup. The photocytes (ph b) branch among the homogeneous packing cells (hc). Branches of mitochondrial cells (mc) are also evident.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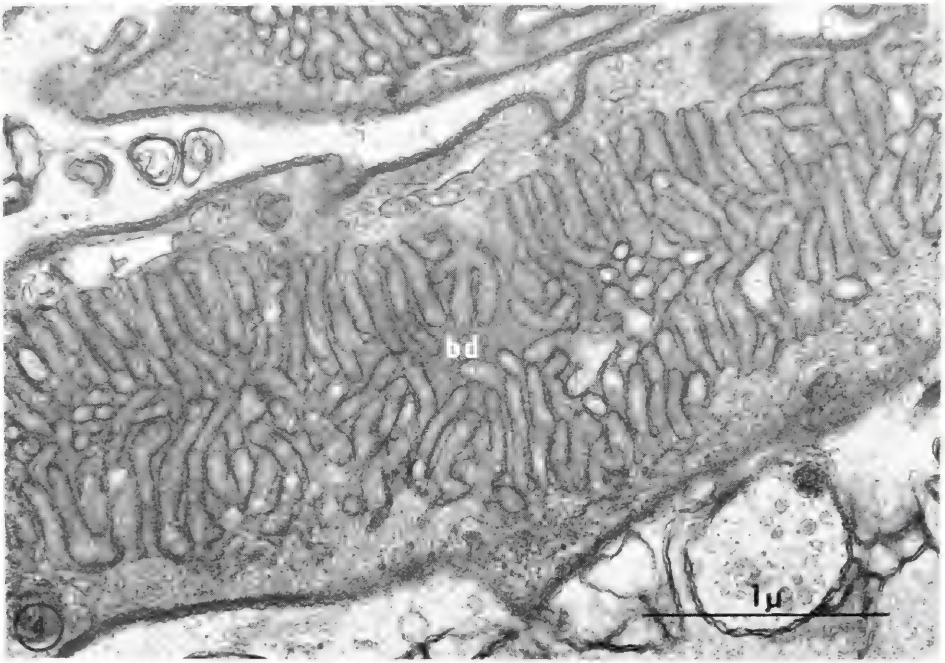

④

MATERIALS AND METHODS

The animals used in this study were taken at night by a closing trawl off the island of Oahu, Hawaiian archipelago. Once on shipboard they were fixed in a mixture of 6% glutaraldehyde buffered to pH 7.5 with 0.1 M collidine buffer and adjusted to approximately 1200 milliosmoles with sucrose as modified from Bell, Barnes and Anderson (1969). Fixation began at ambient temperature and then the vials containing the whole animal were chilled on ice and kept at 4° C until the ship returned the next morning. The photophores were then dissected off the surface of the eye, washed in cold collidine buffer for 1 hour and then post-fixed in 1% OsO_4 in veronal-acetate buffer at pH 7.6 for one-half hour. The photophores were then dehydrated through an ethanol series and then embedded in Luft Epon for thin sectioning. These sections were stained with either uranyl acetate in methanol followed by lead citrate or in lead citrate alone. This fixation procedure seemed to be adequate despite the difficulties caused by working on shipboard, and the time delays involved. However, in some areas there are membrane whorls that are probably artifact. Despite slight variations in fixation procedures these whorls occasionally remained randomly associated with membranous structures in the cells. Because of the difficulty in obtained animals (one or two per night is an average catch), the expense of ship time, and the otherwise satisfactory fixation, the results are reported with no further apology.

OBSERVATIONS

Figure 2 shows a low power micrograph of the "spherical knob" and most of the "axial cone" region of the photogenic tissue. The photogenic tissues are surrounded by stalks of iridophores which are occasionally penetrated by blood vessels and nerves. These iridophores will be discussed in a separate paper. In addition to elements of circulatory system and the nervous tissue, these are four cell types: the photocytes; their associated sheath cells; the packing cells which occupy most of the volume of the photogenic tissue; and the mitochondrial cells. Each of these cell types will be discussed in turn. Although it has not been possible to examine each of these cells in physiologically active state, by elimination and analogy it has been possible to assign these four cell types functions on the basis of their morphology.

Photocytes

The photocytes are branching, ramifying cells which are interspersed with no apparent pattern between the other cells of the photogenic tissues. They are characterized by an extensive body of microvillous protrusions which occupy most of the volume of the cell and are arranged about a central lumen filled with dense

---

FIGURE 3. Mature photocyte (ph) in the nuclear region. The photocyte is enclosed in a sheath (sc) which separates it from the packing cells (hc). The microvilli (mv) are quite evident.

FIGURE 4. Higher magnification of the microvillous region of one branch of a photophore. The microvilli project into a central blood filled lumen (bd). The sheath can be seen to be of homogeneous density and lined by membranes of both sides.

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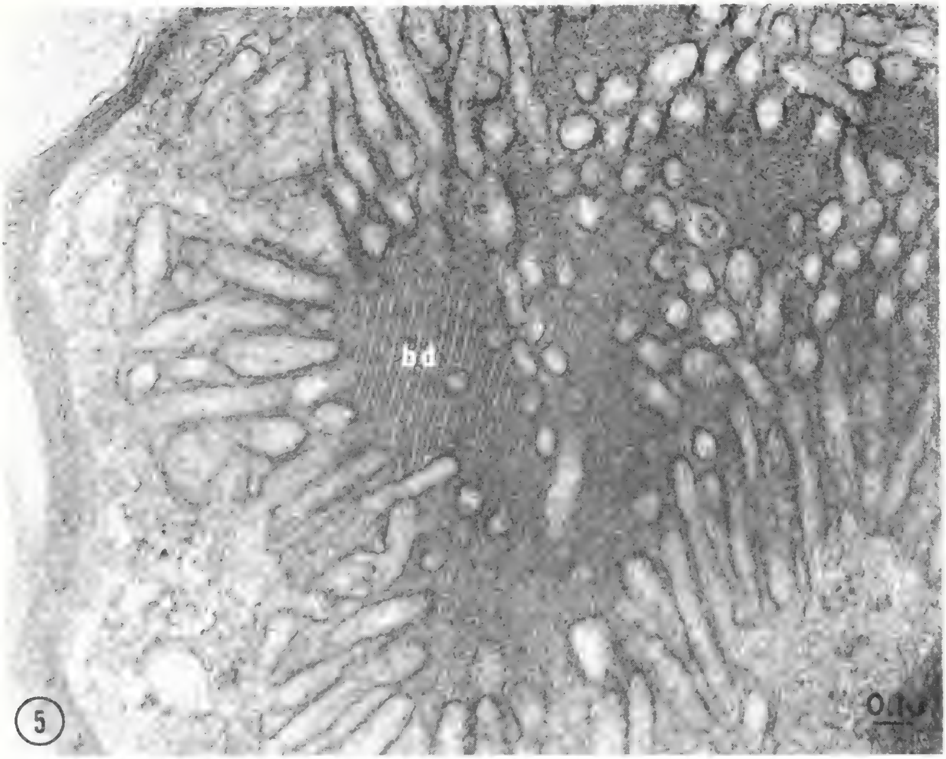

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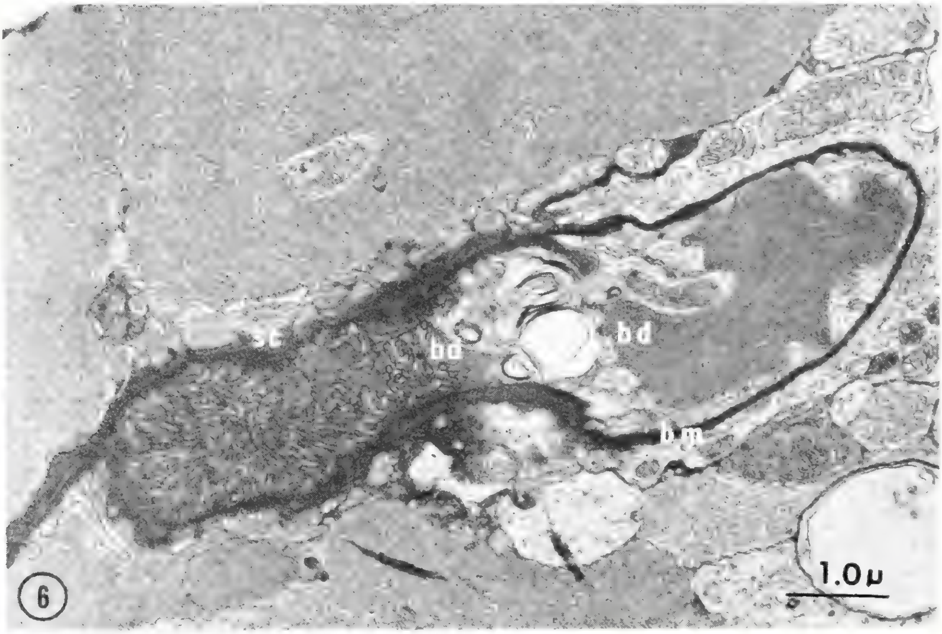

sc
bd
bd
bm
1.0μ

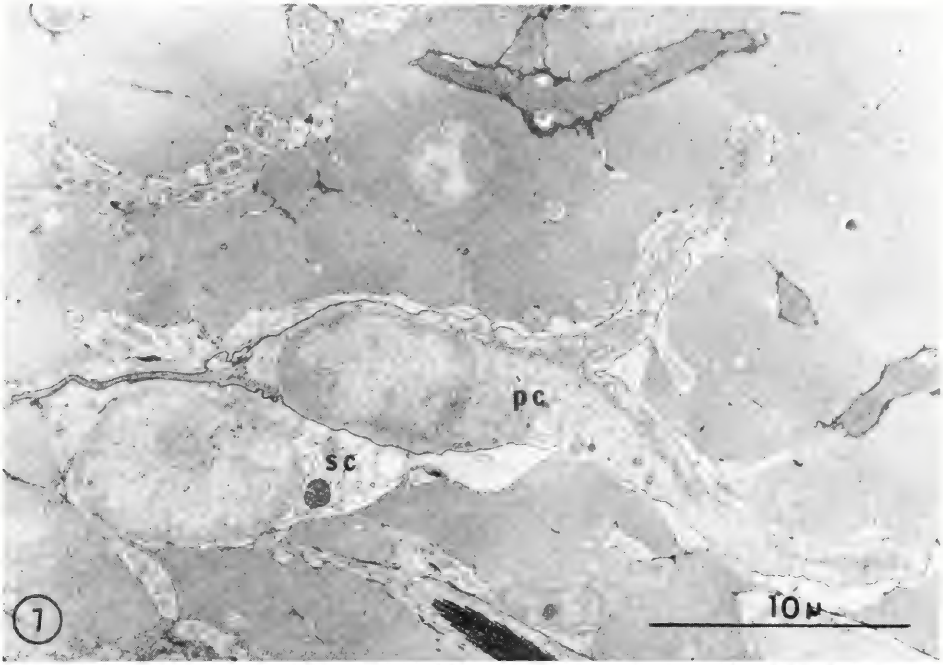
material (Fig. 3). Surrounding each photocyte is a dense sheath of acellular homogeneous material which separates the cell from either the surrounding packing cells or engulfing sheath cell (see below). In sections the nucleus of the photocyte appears more or less homogeneous when compared, for example, with the nuclei of the packing cells or the sheath cells.

The microvillous bodies comprise a discrete part of each photocyte and when apparently mature, are easily distinguished by their electron density. The individual microvilli are cylindrical and have a rounded end. They average 0.1μ in diameter and vary in length from 0.4μ to 0.6μ (Fig. 4). The microvilli project into a central lumen which apparently is filled with blood (Fig. 5). Cephalopod blood frequently has a characteristic paracrystalline appearance when fixed for electron microscopy (Barber and Graziadei, 1965) (Fig. 6). The hemocyanin molecules tend to form chains, each unit of which measures approximately 24 nm in diameter. Cut at various angles other characteristic spacing patterns are evident (Fig. 5) so blood can often be distinguished from electron dense substances. However, the blood does not always fix in this paracrystalline pattern but in these instances other characteristics (basement membrane, homogeneous density) can be used to distinguish blood from other materials of similar density. The microvillous body can often be seen to be continuous with blood vessels and in a few instances it appears that the microvilli are bathed by blood directly from the capillaries (Fig. 6). The sheath which surrounds each photocyte often appears continuous with the basement membrane of the blood vessel.

In the region of the axial cone photocytes which appear to be in stages of development can frequently be found. These immature photocytes are characterized by complete enclosure in a sheath cell, few and less dense microvilli in poorly organized discontinuous microvillous bodies, and by areas of somewhat less dense cytoplasm between the microvilli and the sheath (Fig. 7). As the microvillous body develops, this cytoplasmic area is mostly replaced by microvilli and what cytoplasm remains becomes increasingly dense (Fig. 8). Concommitant with this increase in density, the surrounding sheath cell apparently is retracted leaving the branches of the photocyte surrounded by a dense homogeneous sheath of variable thickness which is continuous with the basement membrane of the circulatory system. The microvilli apparently originate in conjunction with membranous components of the cytoplasm (possibly endoplasmic reticulum) and are frequently associated with infolding of the photocyte surface (Fig. 9). In some instances blood can be found contained within membranous networks within the cytoplasm which appear to be giving rise to microvilli. The origin of the intercellular membranous complexes containing this blood is uncertain but frequently endoplasmic reticulum and Golgi vesicles are seen in close proximity (Fig. 10).

FIGURE 5. Central region of a photocyte showing blood (bd) in the lumen. The hemocyanin molecules align to form paracrystalline arrays, some of which have a chain-like appearance. Sectioned at different angles the pattern is still characteristic enough to make identification of blood possible.

FIGURE 6. Connection between a photocyte and blood vessel. The paracrystalline pattern is evident (bd) in the vessel and the lumen of the microvillous region. The basement membrane (bm) is continuous with the sheath (sc) of the photocyte. The endothelial cell and pericyte are also evident.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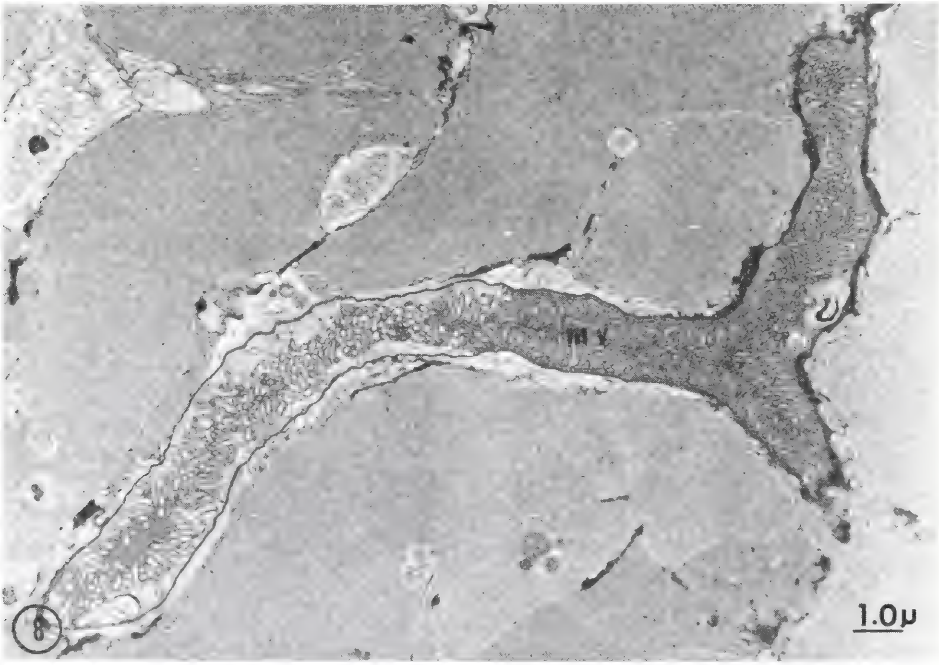
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Sheath cells

The sheath cells characteristically surround the whole developing photocytes encasing them in a layer of cytoplasm of varying thickness (Fig. 7). The nucleus of the sheath cell invariably occurs in proximity of the photocyte nucleus. The cytoplasm is considerably less dense than that of the photocyte and contains few scattered organelles. Mitochondria and membranous vesicles are occasionally encountered but mainly the cytoplasm is homogeneous with small aggregations of granular "background" material. Where the sheath cell contacts a blood vessel, vesicles suggestive of pinocytosis are apparent. The nuclei of the sheath cells are also less dense than those of the photocyte and appear to have fewer regional densities (Fig. 7).

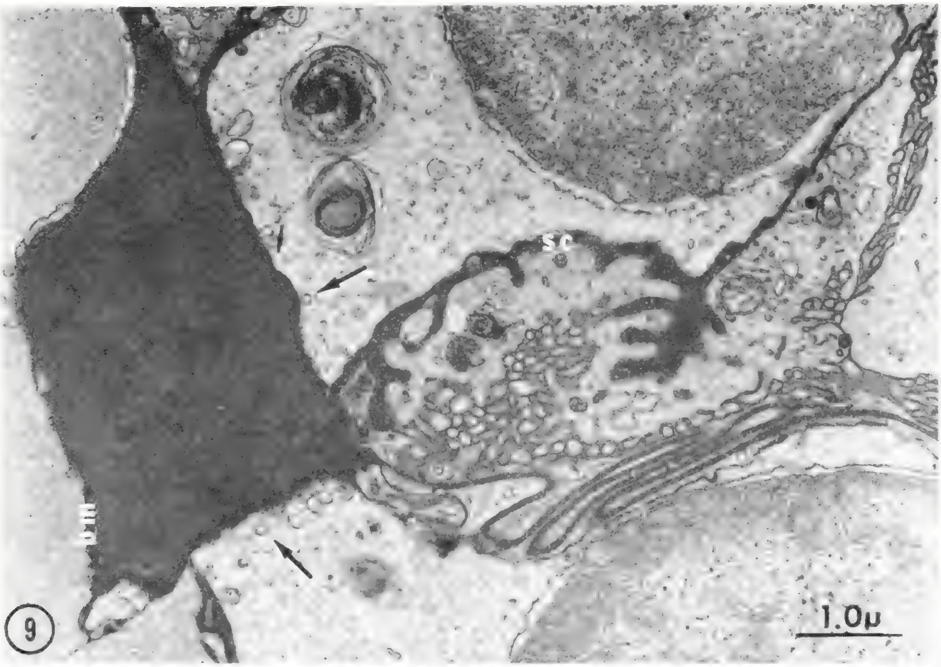
The sheath itself (Figs. 4, 8, 9) is homogeneous, amorphous, and electron dense. It appears to be entirely extracellular because it occurs outside the plasma membrane of both the sheath cell and the photocyte. It can be seen to be continuous with the basement membrane of the blood vessels although it is usually of less uniform thickness. On mature photocytes, it is quite irregular in thickness and shape (Fig. 8). The sheath cells are quite similar in appearance to the pericytes of the blood vessels (Barber and Graziadei, 1965) and in some instances it is hard to separate them on morphological criteria other than position (Fig. 6). As the photocyte matures the association of the sheath cell and photocyte is ended apparently by retraction of the sheath cell so that the photocyte with its surrounding sheath comes into direct contact with the surrounding packing cells (Fig. 8). The eventual fate of the sheath cell could not be determined from our micrographs.

Packing cells

The packing cells are characterized by being filled with a homogeneous granular matrix which occupies the position of the cytoplasm in a more typical cell (Fig. 11). The membranous components of the cell are in direct contact with this matrix and there is no evidence that the matrix is contained in a special vacuole. Occasionally mitochondria are encountered but they appear to be randomly distributed and the outer mitochondrial envelope is in direct contact with the matrix (Fig. 6). Golgi material and a few membranes suggestive of smooth endoplasmic reticulum are present infrequently (Fig. 6). The nuclei have large regions of dense material which primarily occupies the periphery of the nucleus. The packing cells tend to be globular in shape but assume the shape of the more highly structured elements around them.

FIGURE 7. Developing photocyte within a sheath cell (sc). Note that the photocyte is completely enclosed within the sheath cell.

FIGURE 8. Retraction of the sheath as the photocyte matures. The microvilli (mv) completely fill the mature portion of this photocyte branch and the sheath cell no longer surrounds this area. Where the microvilli are less dense and not as well differentiated the sheath cell enclosed the photocyte branch.



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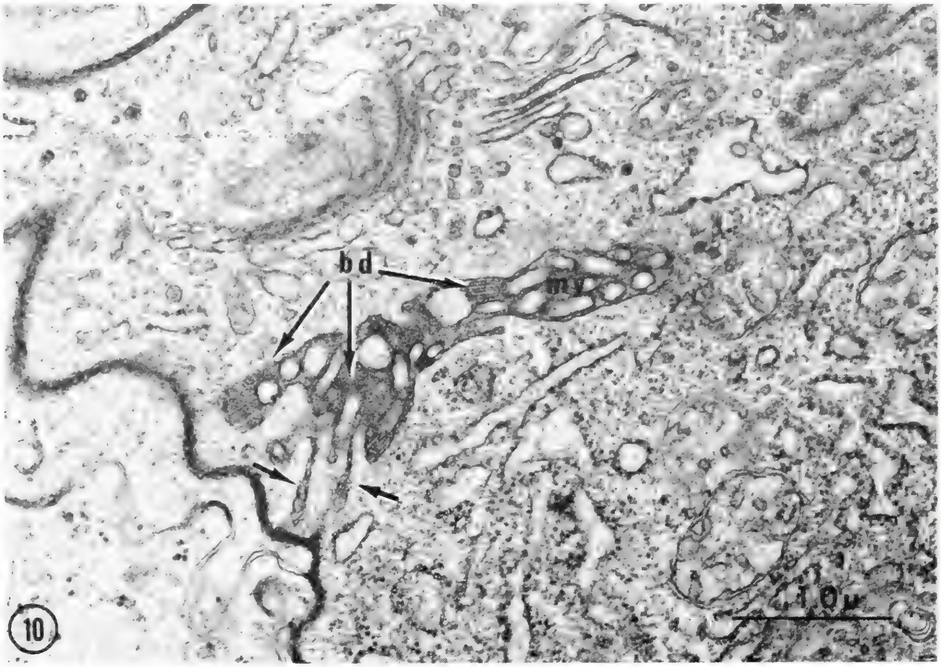

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Mitochondrial cells

In contrast to the packing cells, the fourth cell type in the photogenic tissue, the mitochondrial cells, tend to have their cytoplasm occupied mainly by mitochondria (Fig. 11). These cells tend to be concentrated in the axial cone and in rare cases form a more or less continuous layer. However, because they branch and ramify throughout the photogenic tissue they are present in the spherical knob and are seen in contact with all of the other cell types including the iridophores and no preferential association is obvious. The mitochondrial themselves have the "somewhat empty" appearance typical of cephalopod mitochondria but typically occupy better than 50% of the total cell volume. In contrast to the nuclear condensation seen in the packing cells, the nuclei of the mitochondrial cells seem homogeneous although the cisternae of the nuclear envelope are infrequently expanded (Fig. 11).

Vascular and neural elements

Vascular elements are frequently encountered entering the photogenic tissue. They are easily identified by the characteristic basement membrane, endothelial cells, and pericyte (Barber and Graziadei, 1965). The hemocyanin frequently shows a paracrystalline pattern which also aids in identification (see above). Where vessels pass through the posterior cup, the iridophores are displaced or modified in shape to accommodate them. Inside the photogenic area the endothelial cells and pericytes are frequently reduced or even lacking (Fig. 9), thus allowing the blood to come into intimate contact with the cells of the photogenic tissue. The lumen of the photocytes is frequently seen filled with blood (Figs. 5 and 6) which is in direct contact with the microvilli and occasionally blood can be found within intracellular membranes in developing photocytes.

Neural elements are represented primarily by single nerve processes which occur among the photogenic cells. These nerve fibers apparently form synapses with the photocytes and in favorable sections gaps in the sheath surrounding the photocytes can be found which suggest the possible transport of synaptic vesicles (Fig. 12). We have not encountered any synapses with other cell types of the photogenic tissue.

DISCUSSION

Although the inability to maintain living material in the laboratory imposes certain limitations on the interpretation of the observations presented here, it is possible to speculate on the function of the various cell types presented here on purely morphological grounds. The photocytes seem to be the only possible

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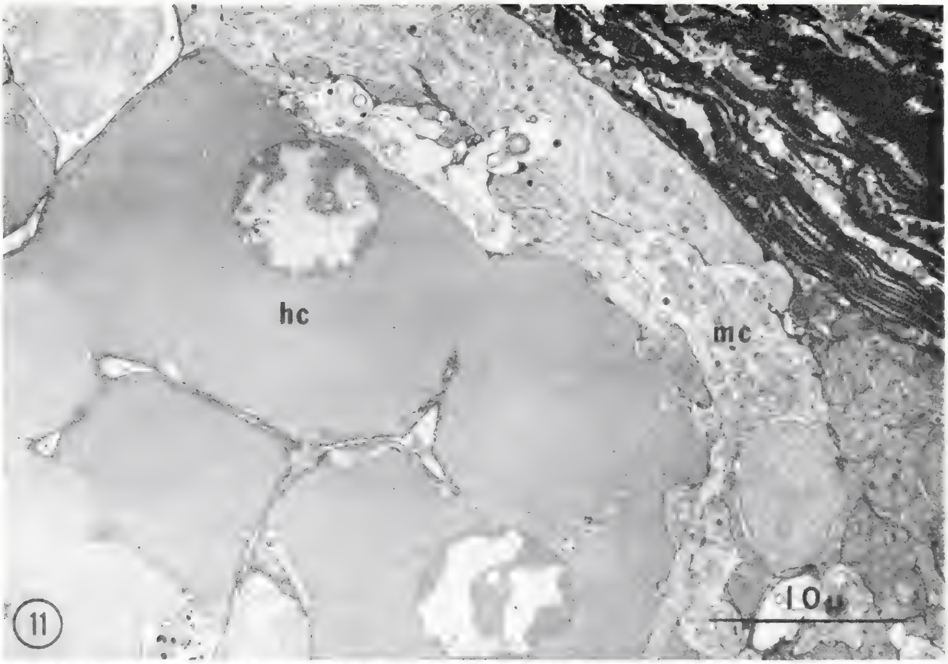
FIGURE 9. Developing microvilli and acellular region of blood vessel. The developing microvilli arise inside the photocyte but are in contact with the surface. A small acellular blood vessel is in contact with the sheath cell and developing photocyte. The sheath (sc) is continuous with the basement membrane (bm). Blood is evident inside the cell in the future lumen.

FIGURE 10. Developing microvilli (mv). Note the membrane continuity between the future microvillous region and the surface (arrows). Blood is clearly evident.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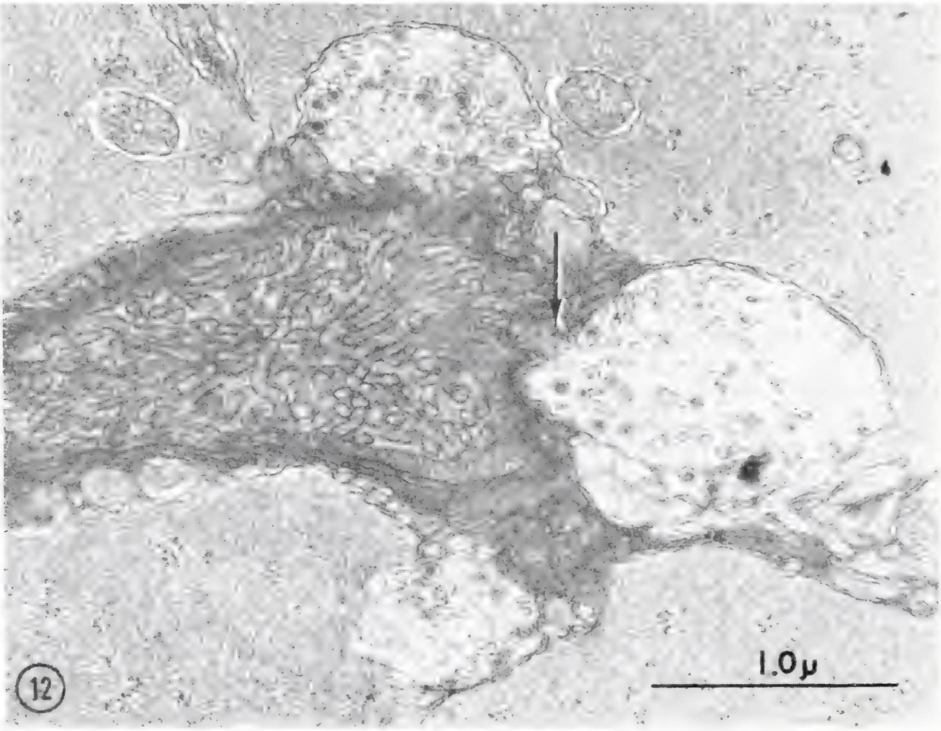
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candidate for light production since they are in the proper position, are highly specialized, and have direct communication with both the nervous system and circulatory system. The microvillous areas of these cells would seem to be the most likely site of light production. In many invertebrate photoreceptors, light is converted to chemical energy in strikingly similar structures (Clark, 1967). The availability of nutrition and oxygen, innervation, and mitochondrial energy would strongly support this assumption. The possibility that the "photophore" might be some type of a secondary photoreceptor could be raised but in other genera organs with quite similar structure are undoubtedly photoproductive (Berry, 1920a, 1920b; Hoyle, 1902, 1904). The morphological similarities between organs of photoreception and photoproduction imply that interconversion of light and chemical energy may have a necessary basic subcellular mechanism.

Although the observations on the development of the photocyte are not sufficient to reconstruct the complete pathway of development a few interesting points may be made. Apparently early in the life of a photocyte, it must be isolated from surrounding tissues; first by developing within another cell (the sheath cell) and later by being enclosed in a dense sheath continuous with the basement membrane of the circulatory system. This would imply that insulation from other cell types is necessary for either photocyte differentiation or function. It would be tempting to speculate that the sheath (and sheath cell) chemically or electrically insulate the photocyte but more likely is the possibility that the sheath functions in keeping the photocyte in intimate preferential contact with the circulatory system thereby insuring an adequate nutrient and respiratory supply. The microvilli of the photocyte apparently arise from an intracellular membranous system that very early in development is in direct contact with blood. The retraction of the sheath cell as the photocyte presumably matures implies that the control of differentiation of the photocyte is somehow directed or enhanced by the sheath cell.

The simple structure of the packing cells suggest that they primarily function in occupying space between more metabolically active tissues. In formalin preserved, unstained photophores, the packing cells are clear and would thereby not impede or modify light produced by the photocytes. It would seem most likely that the packing cells function in dispersal and separation of the photocytes and have a low metabolic level.

Conversely, the mitochondrial cells, by the sheer volume of mitochondria seem to be very metabolically active. Because they branch and ramify throughout the photogenic tissue and make contact with many cells, they probably provide metabolic energy to the other cell types and in particular the photocytes.

The structure of the circulatory system in the photogenic tissue is interesting because blood comes into direct contact with the photocytes and because in some areas vessels are apparently reduced to a basement membrane containing blood with neither pericytes nor endothelial cells present. Barber and Graziadei (1965)

FIGURE 11. Packing cell and mitochondrial cell. Most of the cytoplasmic region of the packing cells (hc) is occupied by a homogeneous material. In the mitochondrial cell (mc) most of the cell's volume is devoted to mitochondria.

FIGURE 12. Synapse with the photocyte. Note the sheath is broken in one region and the synapse appears to be in direct contact with the microvillous region (arrow).

described four types of blood vessels in the circulatory system of *Octopus* and *Sepia,* two of which had the basement membrane exposed directly to the blood. A logical extension of this would be a completely "acellular vessel" composed of basement membrane only. Such a vessel is shown in Figure 9. These "acellular vessels" are frequently encountered and always are smaller than the cells they contact. However, since there always appears to be a basement membrane except where the blood directly contacts the photocytes this is not a true open circulatory system. In the case of the photocyte the basement membrane (=sheath) contains the whole cell suggesting the photocyte is intimately related to the circulatory system.

It is possible to present an integrated speculative picture of the function of the cell types in the photogenic region of this photophore. Light is produced by energy conversion on the microvilli from precursors provided by the circulatory system and energy derived from the mitochondrial cells. The photocytes are probably stimulated to luminesce by the numerous synapses which penetrate the sheath. The photocytes are separated by packing cells and contained within the sheath. Although the above model of light production by one type of photophore of a deep sea cephalopod is hypothetical and theoretically tenuous, it is hoped this model can serve as a tentative structural basis for further comparative and physiological study.

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SUMMARY

One type of photophore of the deep sea squid *Pterygioteuthis microlampas* was examined with the electron microscope and its fine structure described. The photogenic tissue is composed of four cell types each with distinctive morphology which suggests their function. The photocytes branch and ramify throughout the central region of the photophore and have an extensive system of microvilli (the photogenic organelle) which are arranged about a central blood filled lumen. The photocytes apparently develop inside a sheath cell and are surrounded by a sheath which is continuous with the basement membrane of the blood vessels. The photocytes and associated sheath cells are surrounded by packing cells whose cytoplasm is replaced with a homogeneous granular material. Finally, cells containing many mitochondria branch and ramify throughout the photogenic area. Apparently the circulatory system is in direct contact with the photocytes, and acellular blood vessels, composed only of basement membrane, are found throughout the photogenic tissue. The similarity between photoproductive organelles and photoreceptive organelles is striking.

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ULTRASTRUCTURE OF A CEPHALOPOD PHOTOPHORE. II. IRIDOPHORES AS REFLECTORS AND TRANSMITTERS

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Animals that live in the deep sea confront predation pressures concerned with background illumination (Denton, 1970). When viewed from below a pelagic animal at moderate depth casts a shadow which makes it clearly visible to any potential predator below it. In many fishes the problem is, in part, solved by the evolutions of highly reflective sides which reflect light efficiently enough so that it matches the background illumination except when viewed from directly below. This latter problem is frequently diminished either by the fish becoming laterally compressed so it presents a minimum outline when viewed from below, or by the development of bioluminescent countershading which duplicates the background illumination. A third potential solution, that of evolving transparent tissues, is only partially successful because some tissues must be opaque to function. For example the eyes must receive light from only one direction or, in the cephalopods, the ink sac by its very nature cannot be transparent.

Any organism which uses light as a countershading device has to match the physical characteristic of the background illumination. Daylight surrounding an animal swimming at a depth of a few hundred meters is limited in spectrum to the blue band because of the absorbtion characteristic of water. Furthermore, downwelling light is very directional and varies in intensity in relation to depth. For a bioluminescent countershading mechanism to be effective the photogenic organs must be able to simulate these light conditions. Recently Young (1972) has described mechanisms of countershading control involving feedback from extraocular organs of the cephalopods. This paper describes the iridophores of the photophores of *Pterygioteuthis microlampas* and offers speculation of how they might function in effective control of bioluminescent countershading.

Methods and Observations

The techniques used in this study were described in our previous paper (Arnold and Young, 1974). The light microscope observations on freshly dissected photophores were made on shipboard in a darkroom.

The iridophores in the photophore of *Pterygioteuthis microlampas* are of three major kinds with one additional kind occasionally encountered in the anterior capsule region. In addition, a reddish-brown pigment surrounds most of the posterior capsule and is evident even in 1 μ thick sections. Each of these iridophore types is individually described below and the brown pigment is briefly included for the sake of completeness. Based on their morphology and location, the iridophores can be given descriptive names. The anterior and posterior cap iridophores are

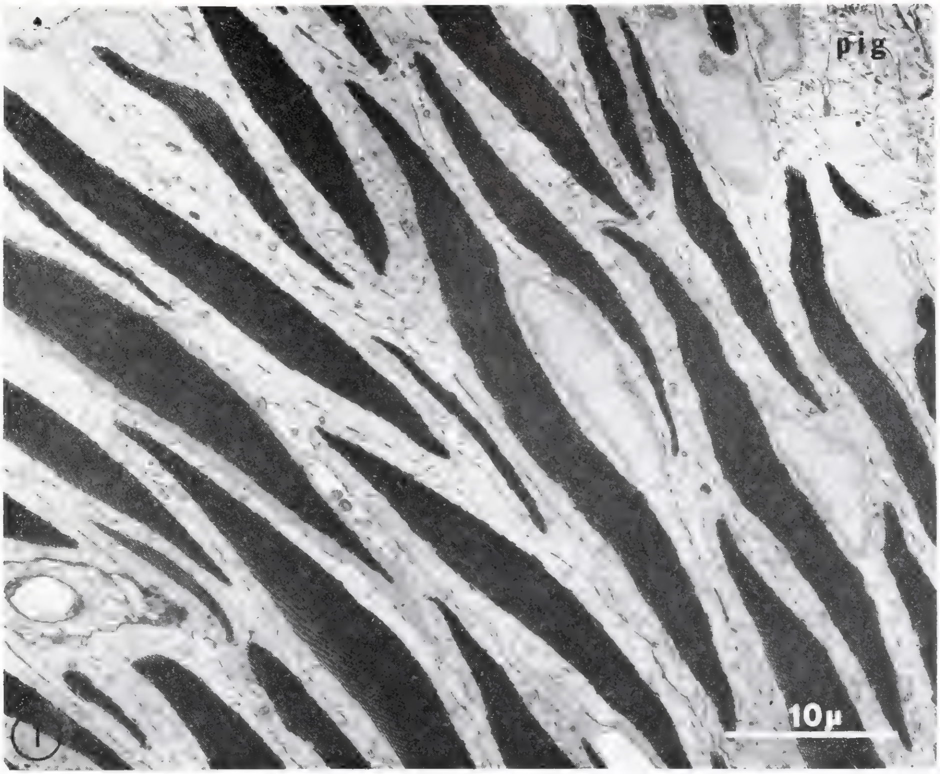
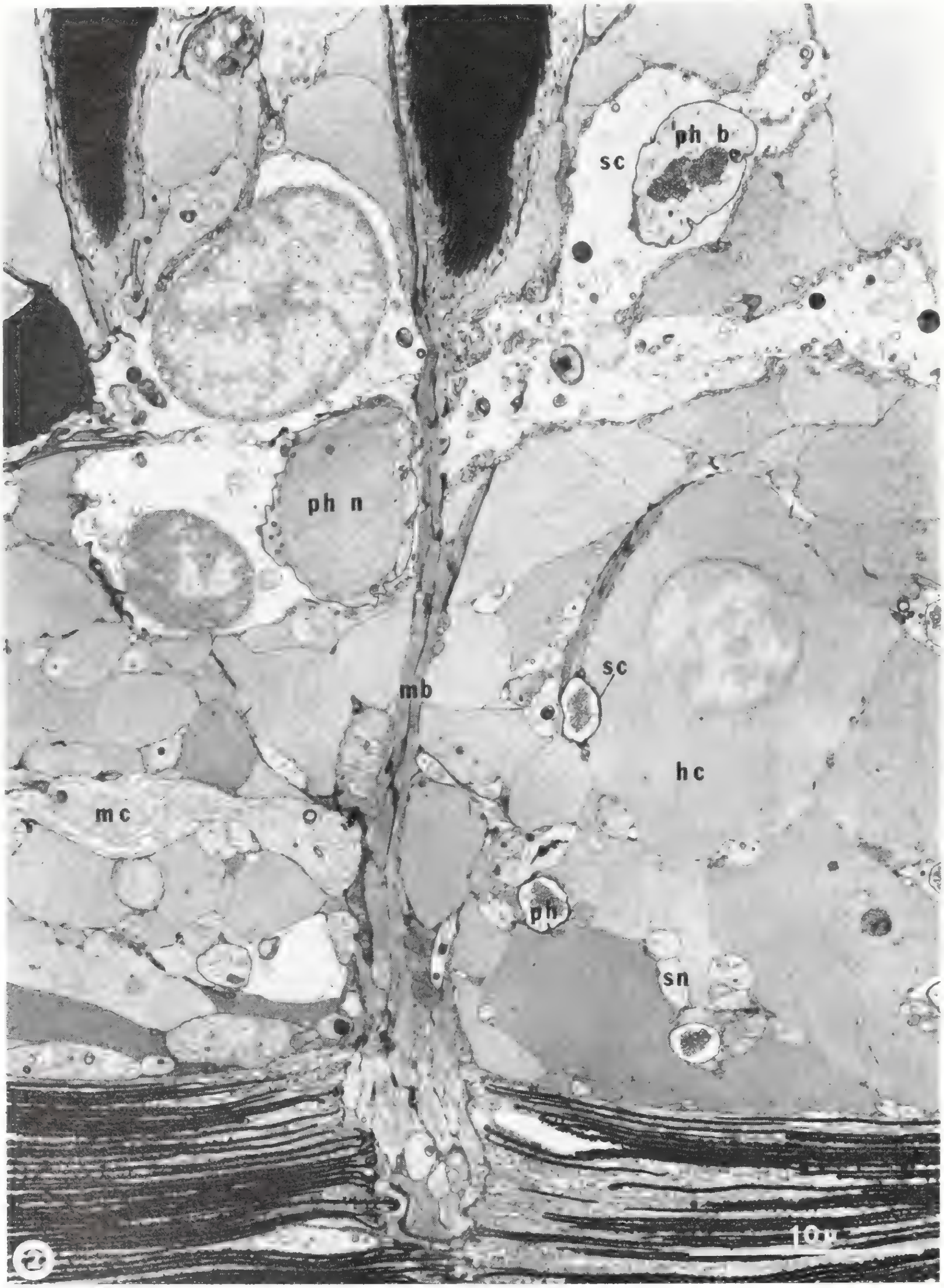


FIGURE 1. Posterior cap regular iridophores showing the single iridosome within each iridophore. The iridophores are separated by a discontinuous layer of intercellular substance. Some of the brown pigment spicules are evident in one corner (pig).

referred to here as "regular iridophores"; those of the inner funnel are called "irregular iridophores" (occasionally they also are found in the skin outside the photophore); and those in the clear lens are called "transparent iridophores" because they transmit light. The other terminology used here is that of Arnold (1967).

Regular iridophores

The regular iridophores are characterized by a highly ordered layering of the individual iridosomal platelets which alternate with extracellular spaces. The platelets are very constant in thickness as is the space between them. The platelets average 75 nm in thickness (range 69 nm–90 nm) and the space between platelets averages 53 nm (range 34 nm–66 nm). The platelets run straight for long distances and follow the general shape of the cell, being slightly curved in the posterior cup or wavy in some regions of the anterior cap. There is one iridosome per iridophore, and it occupies almost the entire volume of the cell (Fig. 1). When viewed with a dissecting microscope individual iridocytes can be teased out of the posterior cup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and appear like silvery mirrors which reflect rather than transmit light. Each regular iridophore is joined to its neighbors by an interrupted dense intracellular substance. The spindle-shape cells overlap to form a continuous layer in the posterior capsule or a thick lentoid block in the anterior capsule. In the center of the axial cone, individual iridophores are stacked more or less above one another. Each of the axial cone iridophores terminates with a muscle junction which continues across the axial cone through the inner funnel and attaches someplace outside the posterior capsule (Fig. 2). Similar strands of muscle also are seen between the iridophore and the rest of the anterior cap but not in the posterior cup region.

The platelets are remarkably parallel and maintain constant spacing over the entire length of the iridosome. At their ends the platelets seem to end in an oblique line rather than all at right angles to their collective long axes, hence the iridosomes are trapezoidal or lentoid in shape (Fig. 1). Each platelet is membrane bounded and these membranes appear to arise by fusion of anastomosing vesicles which originate in the cytoplasm of the iridophores and are continuous with the extracellular space as described in *Loligo* iridocytes (Arnold, 1967). The platelets themselves apparently are formed by the coalescence of dense granular material first into droplets which fuse to a discontinuous reticulum, then into a solid platelet (Figs. 3, 4). The iridosome thus formed is more or less centered in the cell and the nucleus is displaced to one side. The iridophores of the anterior cap are frequently wavy and appear somewhat compressed but still maintain the regular packing between the platelets and intraplatelet space. These iridophores, and in particular, those in the "plug" in the axial cone are separated by a prominent flocculent intercellular materal (Fig. 5). There is a slight but consistent thickness and spacing difference between the iridosomal platelets of the posterior cup and the anterior cap with the iridophores of the posterior cup having an average thickness of 69 nm (range 69 nm–59 nm) and spacing of 50 nm (range 57 nm–44 nm) while the anterior cap platelets average 85 nm (range 90 nm–78 nm) and are spaced at an average of 57 nm (range 50 nm–66 nm). With respect to their electron density, development, and other morphological respects, the iridophores of the anterior cap and the posterior cup appear to be similar.

Irregular iridophores

In contrast to the regular iridophores, the irregular iridophores are extremely variable in their shape, platelet thickness and arrangement, and interplatelet spacing (Fig. 6). They occur in the inner funnel of the photophore and are occasionally found in the skin outside (usually below) the photophore. The platelets are extremely variable in thickness but generally are thicker than the regulars (average

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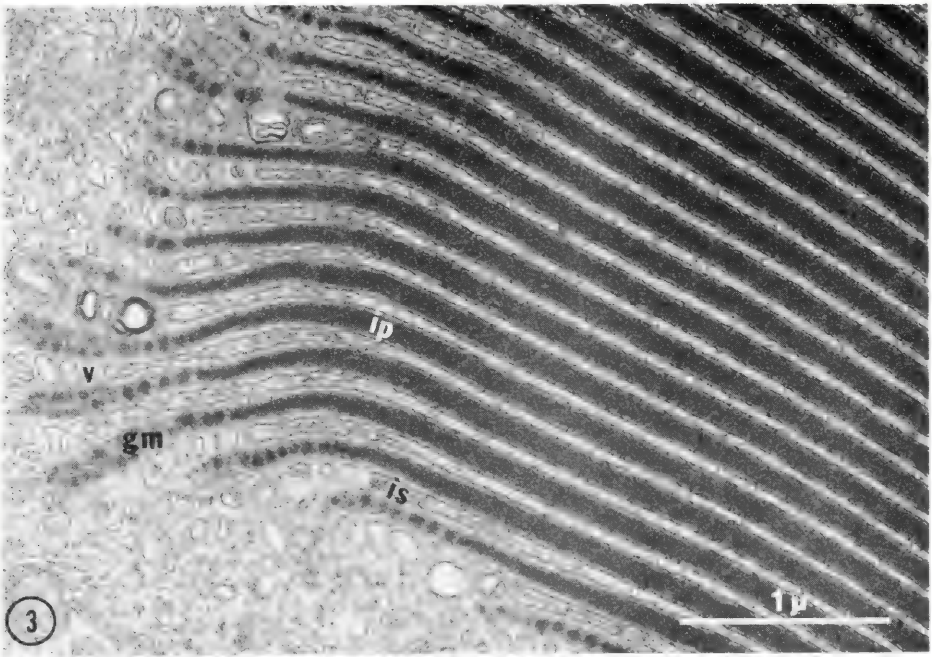
FIGURE 2. Muscle band (mb) traversing the axial cone and irregular iridophore layer. Note the muscle is attached to the regular iridophore of the "plug." The photogenic tissue is composed of several cell types but dominated by a homogeneous packing tissue (hc). Dendritic processes of the photocytes (ph) are surrounded with a dense sheath or a sheath cell (sc). A mitochondrial cell (mc) interdigitates between the packing cells. Within a sheath cell the nucleus (ph n) and one branch (ph b) of a developing photocyte can be seen. Several processes reminiscent of synapses are evident (sn).



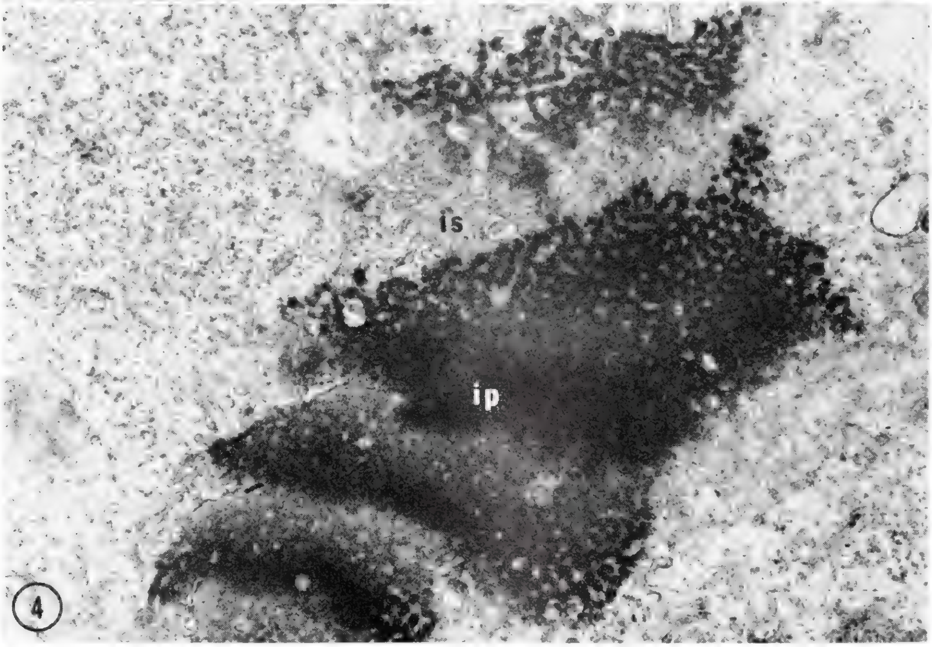


FIGURE 3. End of a regular iridosome showing the formation of the platelets (ip) by fusion of granular material (gm) and the origin of the interplatelet space (is) by fusion of vesicles (v) whose membranes persist as the platelet membrane.

125 nm; range 100 nm–140 nm) and unevenly spaced (average 103 nm; range 58 nm–173 nm). In general the platelets run parallel and in approximately straight lines, but they are covered with irregular knobs which sometimes are hollow or vary in density (Fig. 7). The platelets end in cytoplasmic extensions which themselves are extremely variable in thickness, orientation, and shape. Occasionally microtubules can be seen associated with the end of the platelets in a fashion similar to the development of *Loligo* iridophores (Arnold, 1967). These iridocytes interlock, overlap, and in general are not delineated from each other so that an exact determination of the boundaries of each cell can be made. In general they surround the axial cone to form another cone (inner funnel) which is much thicker at the distal end than the proximal. The general orientation of the platelets is somewhat perpendicular to the abutting concentric regular iridophores of the posterior cup and those of the distal anterior cap. The inner funnel is frequently traversed with muscle strands which connect to the innermost anterior cap iridophores (Fig. 2).

The platelets of the irregular iridophores appear to arise by fusion of granular material and interplatelet space seems continuous with a vesicular network in the cytoplasm of the iridocyte as well as the extracellular space (Fig. 6, 7).

Transparent iridophores

Distal to the anterior cap of the photophore there is a transparent flexible layer which will be referred to here as the lens. The outer surface is covered by an epithelial layer with a highly convoluted surface, a dense basement membrane, and a layer of muscle fibers. The inner surface is underlaid by a continuous surface of pavement epithelium. The iridophores themselves are unique because they contain multiple iridosomes in which the platelets are in almost exact parallel register. In cross section each iridophore contains 20 to 50 such iridosomes arranged throughout the cytoplasm (Fig. 8). In section iridophores may contain as many as 51 platelets which probably represent the maximum number. The platelets average 43 nm in thickness (range 39 nm–45 nm) and are spaced at an average of 82 nm (range 73 nm–90 nm). The platelets are membrane bounded and the spaces between them are separated from the cytoplasm by a continuous plasma membrane. The platelets frequently have a discontinuous appearance and seem to be formed by coalescence of large droplets; although no obvious developmental stages of these iridosomes have been found. Most frequently the platelets seem to end in direct contact with the surrounding cytoplasm but there are examples of one end of an individual platelet being capped by a membrane while the other end is continuous with the cytoplasm (Fig. 9). Although it has not been possible to trace the membrane bounded space to a point continuous with extracellular space, by analogy from the regular and irregular iridophores it would seem that such continuity is likely.

Wide spaced iridophores

In addition to the three commonly encountered iridophores mentioned above, a fourth type of iridophore is infrequently encountered in the photophore or surround-

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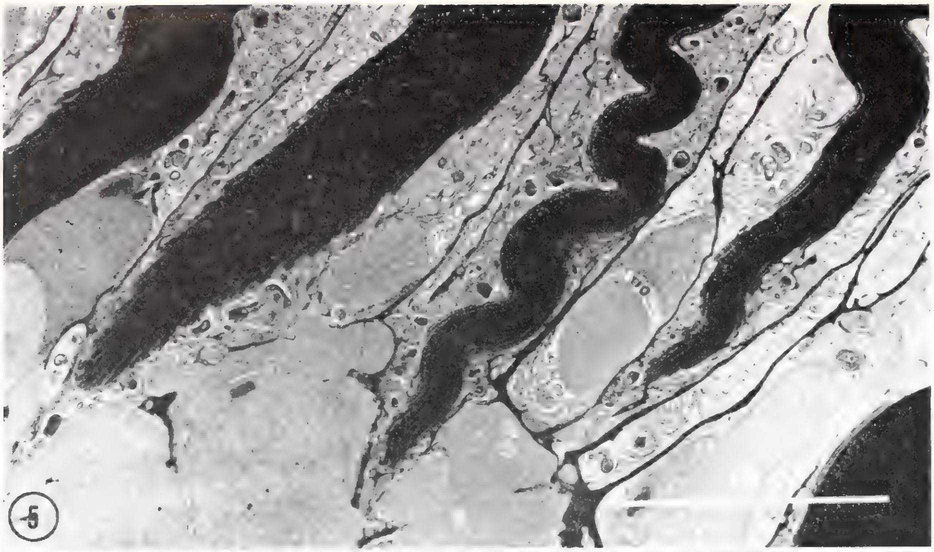
FIGURE 4. Glancing section of several iridophore platelets showing the reticulate nature of the platelets (ip) and the interplatelet space (is).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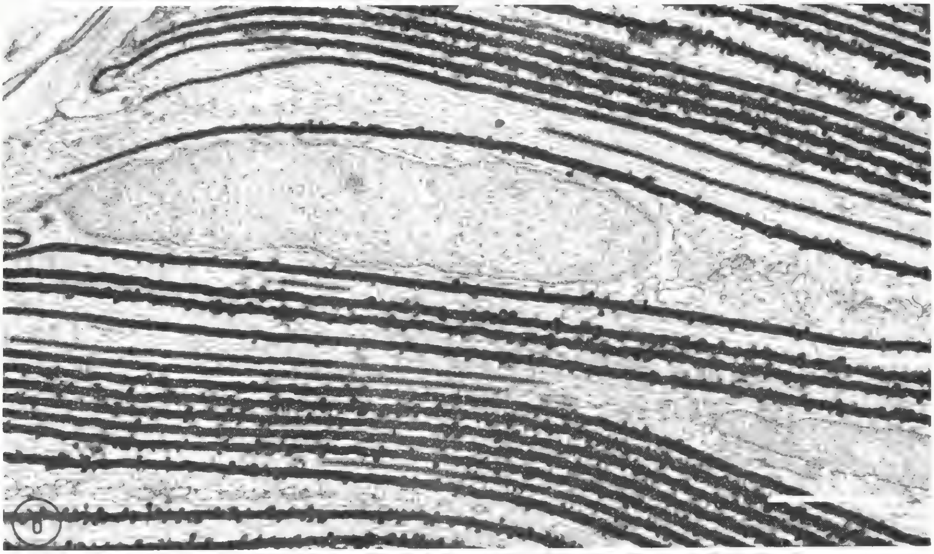
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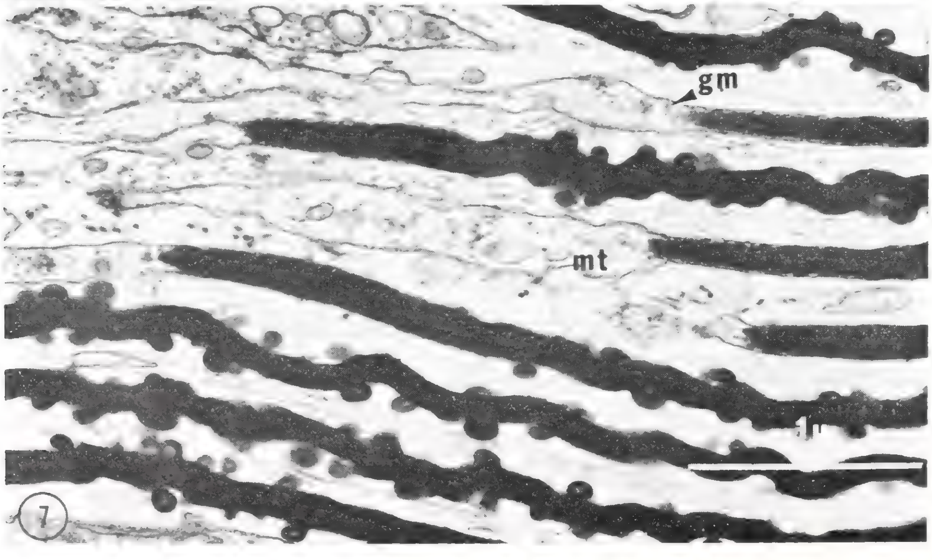
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ing skin (Fig. 11). These iridophores are typified by wide irregular spacing (average 51 nm; range 42 nm–59 nm) and by platelets that follow a somewhat erratic alignment (average 84 nm; range 65 nm–120 nm. The single iridophore occupies a relatively small volume of the cell and the platelets appear to arise by fusion by dense granular material that becomes isolated between the fusing vesicles which form the interplatelet space (Fig. 11). These iridophores rarely occur between the transparent iridophores and the regular iridophores of the anterior cap and are occasionally encountered in the skin outside the photophore proper. Since they are so infrequently encountered, we have no data on their light reflecting characteristics.

Pigmentation of the capsule

In his description of photophores of *Pterygioteuthis*, Hoyle (1902 and 1904) mentioned a reddish-brown pigment layer which is found in the "connective tissue capsule" of the photophore. With the electron microscope, this pigment can be seen to be borne in small intracellular spicules which have a more or less random orientation with the cells (Fig. 12). The outer surface is bounded by a dense layer, but no internal structure is evident within the spicule proper. The pigment does not seem to be borne in any special vacuole or organelle although the cytoplasm between the clusters of spicules is less dense and lacks the granular nature of the rest of the general background cytoplasm. The pigment is dense enough to form a brown layer visible even in 1 μ sections. With polarized light it shows a strong light-blue birefringence in Epon sections. Our micrographs provide no further information as to the origin or development of these brown pigment spicules.

Freshly captured specimens produced a steady glow of blue light which was in a highly directional beam aimed anterioventrally. In freshly dissected photophores the posterior cup iridophores reflected blue to blue-green light normal to the platelets of the iridophore. The irregular iridophores have a "frosted-silver" appearance when viewed at normal angles but at oblique angles are translucent. When illuminated normal to the platelet surface the anterior cap iridophores reflect yellow light and transmit blue and red light. It was extremely difficult to make reflectance observations on the transparent iridophores of the lens because in the intact photophore the reflection of the other iridophores caused confusion. When the lenses were dissected off the photophores they reflected yellow light and transmitted blue at angles normal to the platelet axis.

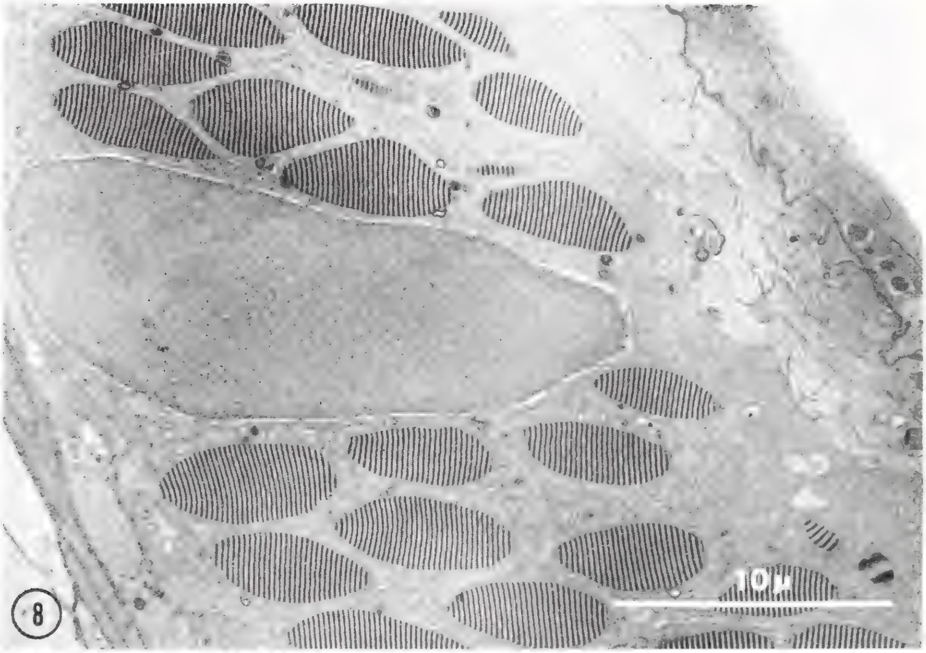
DISCUSSION

The major question to be considered in this paper is the possible function and significance of the various types of iridophores found in the photophore. Denton

FIGURE 5. Iridophores of the "plug" region of the axial cone. Note the iridophores are separated by a dense floccular material and the iridosomes can be convoluted.

FIGURE 6. Irregular iridophores in the layer outside the axial cone. The platelets tend to run in parallel arrays but vary in spacing and thickness and are covered with protrusions.

FIGURE 7. Higher magnification of the irregular iridophore platelets. Microtubules (mt) can occasionally be seen in association with the forming ends of the platelet. The protrusions on the platelet frequently appear to be hollow and seem to be randomly spaced. Fine granular material (gm) appears to be fusing to form the platelets.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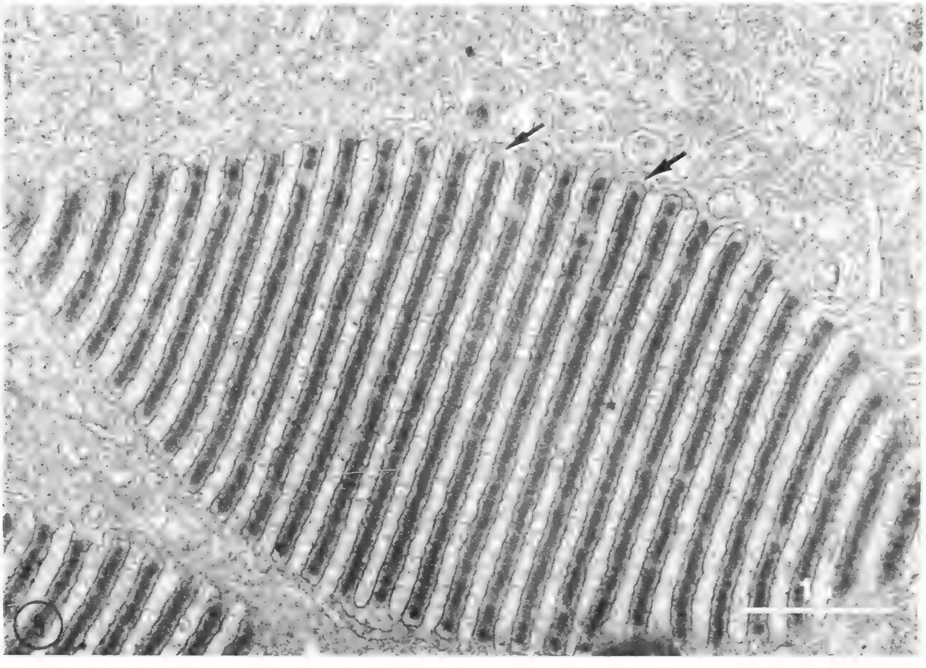
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and Land (1971) have discussed in detail the mechanism of reflectance of iridophores in fish and cephalopods. Huxley (1968) has provided a theoretical basis for the optical behavior of such reflectors, and Land (1972) has summarized both the physical and biological aspects of iridophore reflectance. In both ideal and non-ideal multilayer systems the first-order reflectance peak occurs at $\lambda_{max} = 2\ (n_a d_a + n_b d_b)$ where n_a and n_b are respectively the reflective indices of the optically light and dense layers in the stack, and where d_a and d_b are respectively the thicknesses of these two layers (Land, 1972). We are somewhat hampered in the interpretation of the function of the iridophores because we do not have a direct measurement of the refractive index of either the platelets (optically dense layers) or the spaces (optically light layers) between them. However, Denton and Land (1971) have published values of 1.56 for the refractive indexes of the platelets from the "eyelid" of *Loligo forbesi* and *Sepia elegans*. Following Land (1972) we have assumed a refractive index of 1.33 for the optically light layers. Unfortunately the calculated λ_{max} values based on these indexes and measurements of thicknesses from photomicrographs do not agree with observed reflections from freshly dissected photophores. The most likely source of error is the measurements of the spaces between platelets which may suffer considerable shrinkage during fixation and embedding. Indeed by ignoring the measurements of the spaces and assuming the stacks represent ideal multilayers based on the mean thickness of the platelets, rather close agreement is achieved between observed and calculated reflectance in several cases.

Calculations of reflectance for the regular iridophores in the posterior cup give a λ_{max} of 43 nm which agrees with the subjectively determined blue reflection of freshly dissected iridophores. The orientation of these reflectors suggest that they function in redirecting light that would otherwise be lost. By selectively reflecting blue light these iridophores act as a color filter. Since another color filter is probably present in the anterior cap (see below) this system may seem redundant. However by reflecting only blue light, a light trap (the pigment screen surrounding the posterior cup) is provided for light reflected by the anterior cap iridophores.

Calculations of reflectance for the anterior cap iridophores gives a λ_{max} of 53 nm which agrees fairly well with the subjectively observed yellow reflection. Thus these iridophores will act as a color filter by passing blue light while reflecting probably yellow-green light back into the photophore.

The iridophores of the "plug" region of the anterior cap have somewhat different reflective characteristics. While measurements have not been made on the platelets, observations on fresh photophores indicate they reflect red light. It seems unlikely, however, that the photocytes produce light at these frequencies. These iridophores are attached to muscles which apparently have their origin out-

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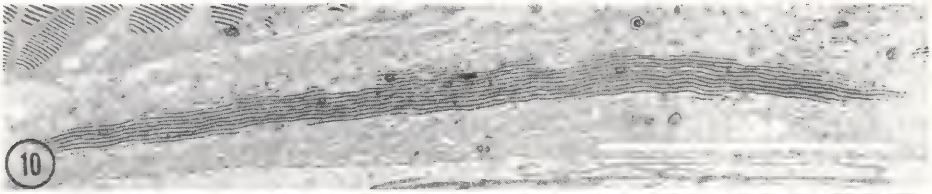
FIGURE 8. Transparent iridophores of the lens. These iridophores contain many iridosomes, each of which has its platelets aligned with platelets in other iridosomes. Thus the whole lens is precisely oriented. The surface of the lens is covered with muscle processes and connective tissue.

FIGURE 9. Higher magnification of a single transparent iridosome. Compare the interplatelet spacing with that of the regular iridosomes. Note that some of the platelets are continuous with the cytoplasm but that others end with a membrane cap (arrows).



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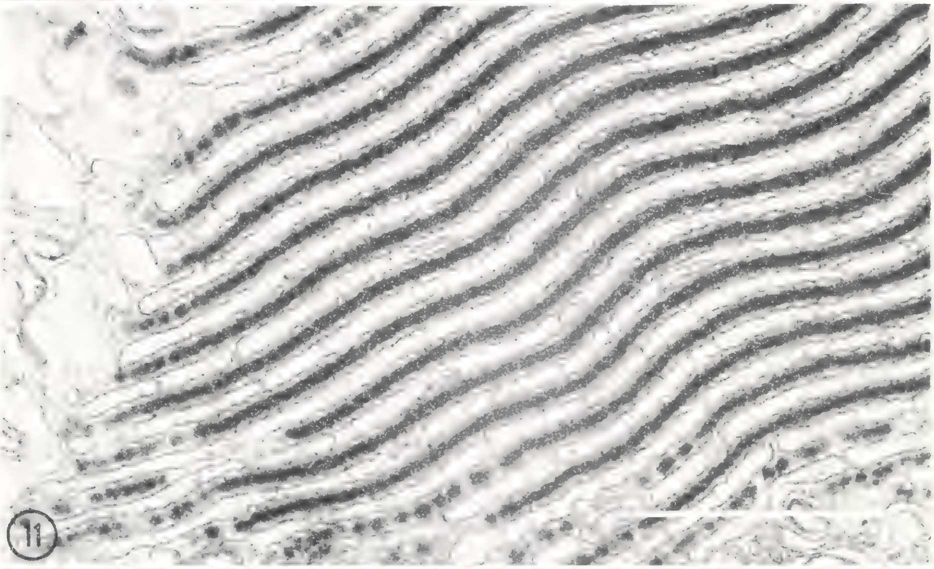

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side the photophore proper. Tensional forces on the periphery of the iridophore from contraction of the muscles may result in reducing the spacing between the platelets thereby altering their transmittance and reflectance characteristics. This may provide a mechanism for regulating the intensity of emitted light perhaps by shifting the reflectance band into and out of the blue region.

The irregular iridophores also offer some interesting ground for speculation. Since the spacing is so irregular and since platelets are covered with protrusions and knobs, they could not possibly function as ideal quarter λ stacks. Their structure suggests they may diffusely reflect light back into the region of the axial cone. In this way light would be redirected but not directionalized as would be the case with the regular iridophores. This assumption agrees well with observations on freshly dissected photophores.

The lens iridophores are very different from the others. All stacks have similar alignment, and they have thin platelets and very thick spaces. These are clearly non-ideal multilayers. If the optical thicknesses of the platelets and spaces have a ratio of 1 : 3 (this assumes approximately the same degree of shrinkage as in the anterior cap and posterior cup iridophores), the calculated reflectance peak is at 54 nm or approximately the same as the anterior cap iridophores. This peak agrees reasonably well with the yellow reflection these iridophores seem to give in fresh photophores. Presumably only blue light reaches the lens iridophores because of the filtering effects of the anterior cap iridophores. Blue light arriving at normal incidence to the lens iridosomes will pass through and out of the photophore. Blue light arriving at oblique angles may be reflected back into the photophores, however the transmittance and reflectance characteristics of these iridosomes require more careful scrutiny. Certainly the precise alignment of the iridosomes suggests they may act to collimate the light.

It would seem, therefore, that the photophore of this midwater squid is a complex organ potentially capable of producing light of controlled wave length in a highly directional beam whose intensity can be regulated. The individual organs are all oriented in the same plane so that a beam of light could be directed vertically downward from the eyes. These organs, therefore, seem to meet all the requirements necessary for ventral countershading.

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FIGURE 10. Wide spaced iridophore. This type of iridophore tends to be wavy and the platelets loosely parallel each other. The iridosome occupies a relatively small volume of the cell. This type of iridophore can be found in the skin or irregularly placed on the photophore.

FIGURE 11. Higher magnification of the wide spaced iridophore. Except for the spacing the iridosome seems similar to the regular iridophore.

FIGURE 12. Intracellular pigment spicules of the brown pigment layer surrounding the proximal posterior cup. The individual spicules more or less parallel the long axis of the tissue layer containing them.

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SUMMARY

The iridophores of one type of photophore of the deep sea squid, *Pterygioteuthis microlampas* were examined with the electron microscope and four different types were found. Three of these types have not been previously described. The regular iridophores of the posterior cup appear to be one-fourth wave length reflectors and redirect the light produced by the photogenic tissue outward. The regular iridophores of the anterior cap have a different spacing and platelet thickness so they apparently pass blue light. The irregular iridophores form a cone around the photogenic tissue and probably randomly reflect light back into the photogenic tissue. The iridophores of the lens have many precisely aligned iridosomes with platelet spacing and thickness so that they appear to collimate light passing through them. It appears that these three types of iridophores reflect, transmit and collimate the light produced in the photophore to match the background illumination hence making an efficient countershading mechanism. A fourth type of iridophore, the wide spaced iridophore, is rarely encountered and probably does not have a significant role in light attenuation in the photophore.

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CLINAL AND SIZE–DEPENDENT VARIATION AT THE *LAP* LOCUS IN *MYTILUS EDULIS*

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The widespread use of enzyme electrophoresis has revealed that almost all populations are characterized by a large amount of genetic variation (Lewontin and Hubby, 1966; Selander, Hunt and Yang, 1969; Ayala, Powell and Dobzhansky, 1971; Gooch and Schopf, 1972; Clegg and Allard, 1972) and the study of many enzymes has indicated that no locus can be predicted *a priori* as being monomorphic or polymorphic. To what extent these polymorphisms are adaptive has not been resolved, and this issue is central to our understanding of how genetic variability is maintained in natural populations.

Evidence for selection may consist of (1) showing a convergence to a characteristic gene frequency after natural or artificial perturbation (Kojima and Yarbrough, 1967; Sved and Ayala, 1970; Berger, 1971), (2) demonstrating a correlation between genic and environmental variation (Koehn, 1969; Schopf and Gooch, 1971; Hamrick and Allard, 1972; Powell, 1971), or (3) finding a progressive change in gene frequencies with increasing age (Koehn, Perry and Merritt, 1971; Tinkle and Selander, 1973; Fujino and Kang, 1968). Deviations from Hardy-Weinberg proportions or persistent linkage disequilibrium may also support a selectionist interpretation (Franklin and Lewontin, 1970; Charlesworth and Charlesworth, 1973; Allard, Babbel, Clegg, and Kahler, 1972; Clegg, Allard, and Kahler, 1972). This study of genic variation at the leucine-amino-peptidase locus in *Mytilus edulis* presents evidence of types (2) and (3).

Mytilus edulis, the blue mussel, has a widespread range from North Carolina to Nova Scotia and is a prolific colonizer of a variety of habitats. Previous studies (Milkman and Beaty, 1970; Koehn and Mitton, 1972) have shown that the species is always polymorphic for three alleles at the leucine-amino-peptidase (LAP) locus and the frequency of these varies significantly from one locality to another. In addition to the three common alleles, [slow (S), medium (M), and fast (F), in order of increasing electrophoretic mobility], at least two other alleles, *R* and *G*, are found in most samples. Milkman (1971) and Milkman and Beaty (1970) have reported that *Mytilus* populations south of Cape Cod generally have a high frequency of the slow allele (around 50%) and those of Cape Cod Bay and northwards have a frequency of 15–30%. Mussels from the Nissequoque River on Long Island have a slow frequency from 11% to 14% (Koehn and Mitton, 1972). Milkman (personal communication) has correlated these variations with differences in the timing of the tidal currents. The LAP genotypes of the *Mytilus* and *Modiolus demissus* populations at the four sites studied by Koehn and Mitton were significantly different between sites, but were similar for the two species at each site. Thus the large scale variation found by Milkman and the smaller scale variation in the Nissequoque River support the hypothesis of selective agency in maintaining this polymorphism.

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Mussels are dioecious and produce pelagic larvae which may disperse to habitats some distance from their parents. The larvae settle and after metamorphosis attach to a firm substrate and to each other by byssae, thus insuring an essentially sessile adult existence. For this reason, if a particular suite of environmental factors favors particular genotypes, we may anticipate a progressive change in gene frequency with increasing size. This study of *Mytilus* populations on the southern shore of Cape Cod Bay shows both clinal and temporal divergence in LAP allelic frequencies.

METHODS AND MATERIALS

Mussels were collected from two estuaries on the northern side of Cape Cod. The first locality, Sandwich Harbor Mill Creek, is 1 km east of the northern terminus of the Cape Cod Canal, and the second, Scorton Creek, is 2.2 km east of Sandwich Harbor. Mussels are found in channels which drain extensive salt marshes and which are inundated by tidal sea water (mean tidal range is 3 meters). *Mytilus* is found in both the subtidal and intertidal zones from the entrance of the

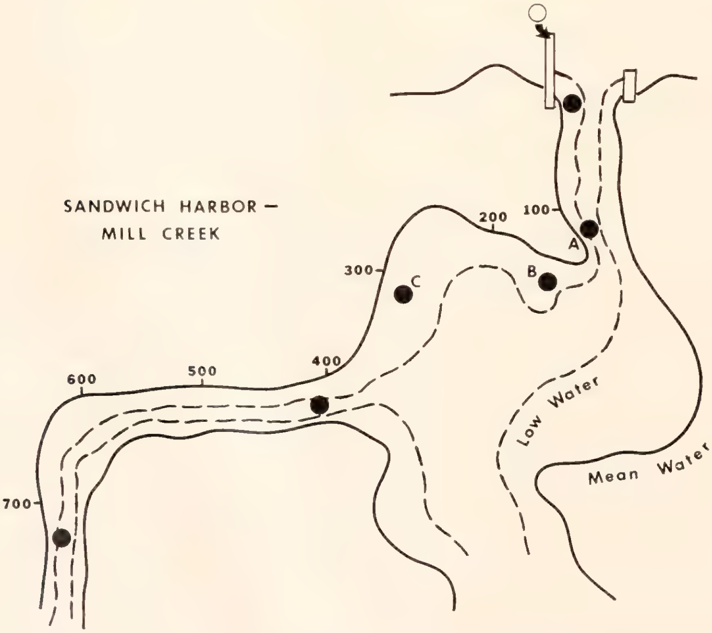

FIGURE 1. Sandwich Harbor. The six sites in the estuary are indicated as filled circles, and the temporary colony from the high intertidal as an open circle. The distance from the entrance measured along mean tide line is given in meters.

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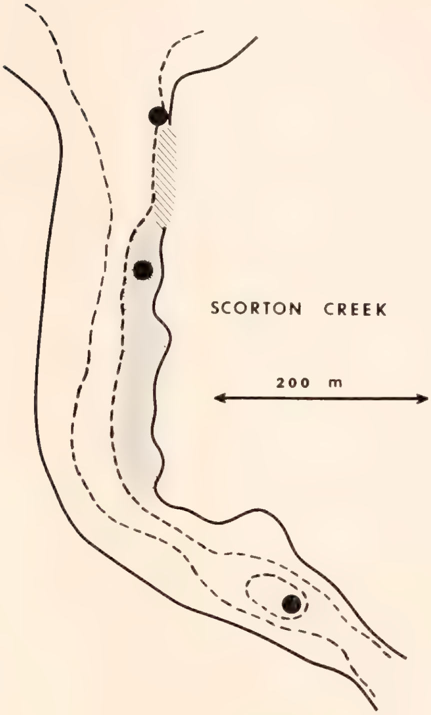

FIGURE 2. Scorton Creek. Filled circles mark the three sites. Stone boulder jetty is indicated by hatching and the extensive mussel beds by stippling.

channels into Cape Cod Bay to a point 800 meters upstream at Sandwich Harbor and 600 meters at Scorton Creek, but are not found higher than mean water. Mussels were sampled from 6 sites at Sandwich Harbor and 3 sites at Scorton Creek (see Figs 1 and 2).

Collections were also made from the intertidal zone at the jetty on the east side of northern terminus of the Cape Cod Canal, the jetty at the Sandwich Harbor entrance, and five sites around the circumference of the very large bay and estuary which comprises Barnstable Harbor (12 km east of Scorton Creek).

Mussels were maintained in the laboratory for one to three days in running sea water. Mortality was negligible prior to assay. The maximum length was measured and then a portion of the liver was homogenized in a 1 : 1 dilution of running buffer (commercial Gelman tris-barbital, pH = 8.8). This extract was spotted on Gelman cellulose polyacetate strips and electrophoresis was carried out in Gelman Trays for 45 minutes at 250 V, 2 mAmp/strip. Strips were incubated for 6 minutes in 25 ml tris-maleate buffer (0.2 M) at a pH of 5.2 (adjusted with NaOH) containing 10 mg 1-leucyl-β-naphthalamide. They were stained for 6 minutes in 25 ml tris-maleate with 25 mg of Fast Black K-salt.

TABLE I

Gene frequencies and sample sizes for all localities by size classes. Number of rare alleles, and observed and expected (Hardy-Weinberg) percentages of homozygotes (SS, MM, and FF) also given.

Size range (in mm)	Number in sample	Frequency (in %) of:			Number of rare alleles	Per cent homozygotes:	
		slow	medium	fast		observed	expected
Sandwich Harbor/Mill Creek							
Mill Creek Upstream							
4–8	143	36	28	36	1	51	34
9–13	98	17	34	49	0	47	38
14–19	138	24	33	43	2	55	35
20–25	133	25	33	42	4	44	35
26–31	96	14	41	45	4	58	39
32–45	209	13	38	49	4	57	40
46–70	214	16	38	46	3	57	38
Mill Creek Subtidal							
5–19	153	16	31	53	4	47	40
20–33	87	22	28	50	0	56	38
34–81	146	15	30	55	3	55	42
Station "C"							
20–25	30	25	34	41	1	—	—
26–31	53	22	32	46	2	47	36
32–56	63	35	27	38	1	45	34
Station "B"							
26–31	30	30	32	37	1	—	—
32–44	66	26	34	40	1	60	34
Station "A"							
12–25	74	24	33	43	1	59	35
26–39	22	49	32	19	1	—	—
Sandwich Harbor Entrance							
9–13	49	24	36	40	1	52	35
14–19	69	18	42	40	4	55	37
20–25	47	53	22	25	1	65	39
26–31	75	54	19	27	1	43	40
32–53	69	47	27	26	3	30	36

TABLE I (*continued*)

Size range (in mm)	Number in sample	Frequency (in %) of:			Number of rare alleles	Per cent homozygotes:	
		slow	medium	fast		observed	expected
Scorton Creek Upstream							
4–8	21	33	24	43	0	—	—
9–19	109	23	33	44	2	51	35
20–25	45	37	30	33	0	60	34
26–45	65	28	26	46	2	35	36
46–76	114	16	34	50	3	47	39
Intermediate							
8–15	96	25	37	38	5	46	34
16–29	96	24	32	44	4	48	35
30–45	95	38	28	34	2	52	34
46–70	75	30	34	36	1	46	34
Entrance							
12–20	89	29	32	39	1	50	34
21–30	20	30	37	33	0	—	—
31–40	35	47	23	30	0	46	36
Two Jetty Sites Cape Cod Canal							
7–13	83	35	25	40	2	54	34
14–19	65	42	22	36	2	51	35
20–25	50	46	29	25	1	47	36
26–31	42	45	17	38	1	58	38
35–82	108	42	27	31	6	50	35
Sandwich Harbor							
3–8	272	38	29	32	10	50	34
9–11	76	21	33	46	4	52	36
12–19	52	30	36	34	2	55	34
Barnstable Harbor/Five Sites Mussel Point							
14–29	79	44	23	32	0	48	35
30–40	63	32	26	42	1	52	35

Table I (*continued*)

Size range (in mm)	Number in sample	Frequency (in %) of:			Number of rare alleles	Per cent homozygotes:	
		slow	medium	fast		observed	expected
Barnstable Harbor/Five Sites Mill Way Bridge							
18–25	31	39	22	39	1	—	—
26–32	19	40	34	26	0	—	—
33–45	50	53	24	23	1	45	39
46–72	50	56	22	22	1	43	41
Scudder's Lane							
20–25	33	33	27	40	0	—	—
26–31	15	47	20	33	0	—	—
32–85	58	45	23	32	3	54	36
Rendezvous Lane							
27–44	50	44	25	31	0	40	35
46–70	50	51	21	28	0	46	38
Barnstable Upstream							
19–39	30	33	18	48	0	—	—
40–78	28	52	21	27	0	—	—

RESULTS

The gene frequencies for the slow, medium and fast alleles for all localities are given in Table I. The number of the two rare alleles is also given; these alleles constitute only 1.1% of the total and show no pattern in their distribution over size classes or sites. Three mussels (in 4871) produced no discernible spots.

The pattern of allelic variation

From Table I we see that mussels in the different size classes from the Sandwich Harbor and Scorton Creek localities exhibit a wide variation in the frequency of S (from 13% to 54%) and a relatively constant ratio of M to F (about 2:3). The smaller mussels from all sites at these two localities generally have intermediate S frequencies, although there are differences between some of the classes at a site (note the contrast among the smallest three classes at the Mill Creek Upstream site). The larger mussels (those greater than 25 mm) show the greatest differences, and both Scorton Creek and Sandwich Harbor/Mill Creek have the *same* pattern of low frequencies of S at the upstream sites and high frequencies of S at the entrance sites. This allelic variation is best summarized by the Chi-square tests of Table II, which compare the numbers of slow alleles among sites by combined size classes. For all mussels 25 mm and smaller, sites are essentially homogenous; the same test applied to the larger mussels reveals striking heterogeneity. Comparison of the larger mussels from the two entrance sites alone shows no significant difference; the two upstream sites are significantly different (S is 15% *versus* 21%), but converge to virtually identical frequencies when only those mussels over 45 mm are compared. The mussels from all the intermediate sites are concordant with this clinal pattern in that there are similar slow frequencies in the smaller mussels and the larger mussels have intermediate frequencies. Size classes between different sites may not be strictly comparable because the growth rates depend on such environmental factors as exposure time and food supply; however the class intervals were chosen *a priori* and not on the basis of tests of significance and therefore the tests and interpretations are conservative.

There is no good topographical counterpart to the Sandwich Harbor and Scorton Creek estuaries at Barnstable Harbor. Three of the sites—Scudder's Lane, Rendezvous Lane, and Mussel Point—were rock/sand beaches on the perimeter of the open water of the Harbor; in this they resembled the estuarine entrance sites, but differed in that they were far from the outlet of the Harbor to Cape Cod Bay. No mussels were found in the channels of the marsh except at the Barnstable Upstream site where they were very sparse; very few mussels were found alive and none was smaller than 19 mm. The Mill Way site was an isolated colony attached to a stone bridge about 100 meters from the Harbor.

The frequencies of the mussels from the Canal Jetty are essentially invariant throughout the size range. The Sandwich Jetty mussels were from a recent and temporary settlement, for no mussels were larger than 19 mm and most (87%) were less than 12 mm. Their growth rates were probably retarded since they were found high in the intertidal.

Although the ratio of medium to fast frequencies is much less variable among samples than is slow allele frequency, the ratio of medium to fast is not independent of slow frequency; the ratio is generally greater than 2:3 in those samples with a high slow frequency, and less than 2:3 where the slow frequency is low. This is true for Barnstable Harbor as well as Sandwich Harbor and Scorton Creek, and this observation may support the hypothesis that these alleles are not a neutral polymorphism.

Deviations from Hardy-Weinberg frequencies

Mussels in almost all size-classes from all localities show a significant excess of homozygote genotypes (SS, MM, and FF) over Hardy-Weinberg expectations. From Table I can be seen that for those samples of 35 or more individuals, only one (from the entrance to Sandwich Harbor) has fewer homozygotes than expected, and the average excess is 14%. There are three general explanations for this which do not involve selection: (1) presence of a null (or silent) allele, (2) persistent inbreeding, or (3) incorporation of the progeny of different populations into one sample.

Table II

Chi-square test for heterogeneity on allelic proportions for Sandwich Harbor/Mill Creek and Scorton Creek localities; by size; A = Mill Creek Upstream Intertidal; B = Sandwich Entrance; C = Scorton Creek Upstream; D = Scorton Creek Entrance (Jetty). Expected values in parentheses; $n_{ij} = n_{i.}n_{.j}/n_{total}$.

(1). Mussels Under 26 mm

	A	B	C	D	Sum
Slow:	267	96	97	54	514
	(262)	(95)	(102)	(56)	
Fast + Medium:	628	228	251	137	1244
	(633)	(229)	(246)	(135)	
Sum:	895	324	348	191	1758

Chi-square = 0.58; 3 d.f.; $P > 0.90$; 514/1758 = 29%

(2). Mussels Over 25 mm

	A	B	C	D	Sum
Slow:	150	144	74	42	410
	(239)	(66)	(83)	(22)	
Fast + Medium:	877	137	281	54	1349
	(788)	(215)	(272)	(74)	
Sum:	1027	281	355	96	1759

Chi-square = 190; 3 d.f.; $P < 0.001$

(3a). Two Upstream Sites (A and C);
Mussels Over 25 mm

	Mill Creek	Scorton Creek	Sum
Slow:	150 (14.6%)	74 (20.8%)	224
	(166)	(58)	
Fast + Medium:	877	281	1158
	(271)	(297)	
Sum:	1027	355	1342

Chi-square = 7.56; 1 d.f.; $P < 0.01$

(3b). Two Upstream Sites (A and C);
Mussels Over 45 mm

Slow:	68 (16.0%)	37 (16.6%)	105
	(69)	(36)	
Fast + Medium:	357	186	543
	(356)	(187)	
Sum:	425	223	648

Chi-square = 0.04; 1 d.f.; $P > 0.80$

TABLE II—(*Continued*)

(4). Two Entrance Sites (B and D);
Mussels Over 25 mm

	Sandwich Harbor	Scorton Creek	Sum
Slow:	144 (51.2%) (139)	42 (43.8%) (47)	186
Fast + Medium:	137 (142)	54 (49)	191
Sum:	281	96	377

Chi-square = 1.61; 1 d.f.; $P < 0.20$

A mussel heterozygous for a null allele will be scored as a homozygote, and if the null allele is lethal when homozygous, only test crosses will detect its presence. Under the hypothesis, the frequency of the null allele (ν) can be estimated as

(1) $(1 - t)/(1 + t) = \nu$, where t is the ratio of observed heterozygotes to expected heterozygotes.

Inbreeding without change in gene frequency will produce an excess of homozygotes with frequency

(2) $\Sigma f(p_i) + (1 - f)p_i^2$, and the total number of heterozygotes will be reduced by a factor f (the inbreeding coefficient, Crow and Kimura, 1970). Adjustment by this parameter reduces the disparity between expected and observed by a factor of about 10.

Both the null allele and inbreeding models produce identical expected frequencies; in fact we obtain

(3) $f = 2\nu/(1 + \nu)$ or $\nu = f/(2 - f)$

and therefore can not be distinguished by the data of a single locus. For most of the samples, estimates of ν range from 7% to 16% (or f from 0.13 to 0.28). These are high values.

The alternative explanation—that individuals in a sample originate from parental populations with disparate gene frequencies (the Wahlund effect)—seems more reasonable because (1) the extended pelagic existence favors dispersal and admixture, (2) striking differences in gene frequencies between adjacent size classes are observed, and (3) there are adjacent localities with characteristically high or low slow frequencies which are likely sources for the immigrants (Milkman and Beaty, 1970; Milkman, 1971).

In this model, we hypothesize that two parental populations produce progeny (with different Hardy-Weinberg proportions) which comprise a mixed population at the sampling site. The six genotypes in the mixed population may estimate five variables (the relative contribution of the parental populations and the frequencies of two alleles in each) and a minimum least-squares fit to the data obtained by trial-and-error methods. However, once this is done, the system is still underdetermined and even if parental populations are assumed to have slow frequencies at or beyond the observed extreme values for slow, this model does not account for the data as well as the inbreeding model. Further, this mixed population model would also predict a decrease in homozygote excess in those size-classes with extreme values of the slow allele. Figure 3 does not show (with one exception) this trend. A further objection is that all other populations of *Mytilus*—including those putative parental populations with a nearly constant high or low slow frequency—show a consistent homozygote excess (Koehn and Mitton, 1972; Milkman and Beaty, 1970).

Environmental variation

Since our hypothesis is that the divergence in slow allele frequency is brought about by selection, we would like to correlate such environmental factors as exposure time, temperature, salinity, current and wave action, substrate, and associated biota with the pattern of genic variation. Environmental differences can be seen to affect the mussels even without electrophoretic assay, because their size range progressively increases as one goes upstream. Extensive search at both entrance sites did not produce any mussels larger than 53 mm at Sandwich Harbor and 40 mm at Scorton Creek. At the upstream sites there were many mussels per square meter larger than 60 mm.

The period of exposure does not seem important because the Mill Creek mussels were taken from both the high intertidal (exposure time in excess of five hours out of the twelve and one-half hour tidal cycle) and the subtidal zones, and the Scorton Creek upstream mussels were from the lowest intertidal (exposure time of about one hour). Mussels at both entrance sites were exposed for two to three hours.

At high tide mussels at all localities were covered by full-strength sea water (salinity of 32‰) and at low water the subtidal mussels were in effluent of minimum salinity of 10‰. The lowest values for sites A, B, and C were 12–14‰ and for the Sandwich Harbor entrance the minimum was 20‰. The Mill Creek Upstream mussels were never exposed to water of less than 26‰. Although the ultimate sources of the channels are fed by fresh water, the extensive marsh stores sea water like a sponge, and therefore the lower salinity values (less than 25‰) are recorded in the stream only after many of the mussels have been exposed. The temperature range of the water follows the same pattern in that the temperature was between 19° and 21° except in the few tide pools where it reached 30°, and in the channel at low tide where a maximum reading of 24° was once obtained on a hot sunny day. Air temperature over exposed mussels was close to ambient.

The chief difference between entrance and upstream sites is that mussels at the entrance were almost always attached singly to rocks buried in the sand, whereas upstream mussels were found in clumps or beds with direct attachment to oneanother. At Mill Creek in particular, the beds were extensive and overlayed an ooze composed of fine silt, organic deposit, and broken mussel shells into which a collector would sink to his knees. At the Scorton Creek Upstream site the mussels were from mid-channel near low water, and had attached to large timbers which

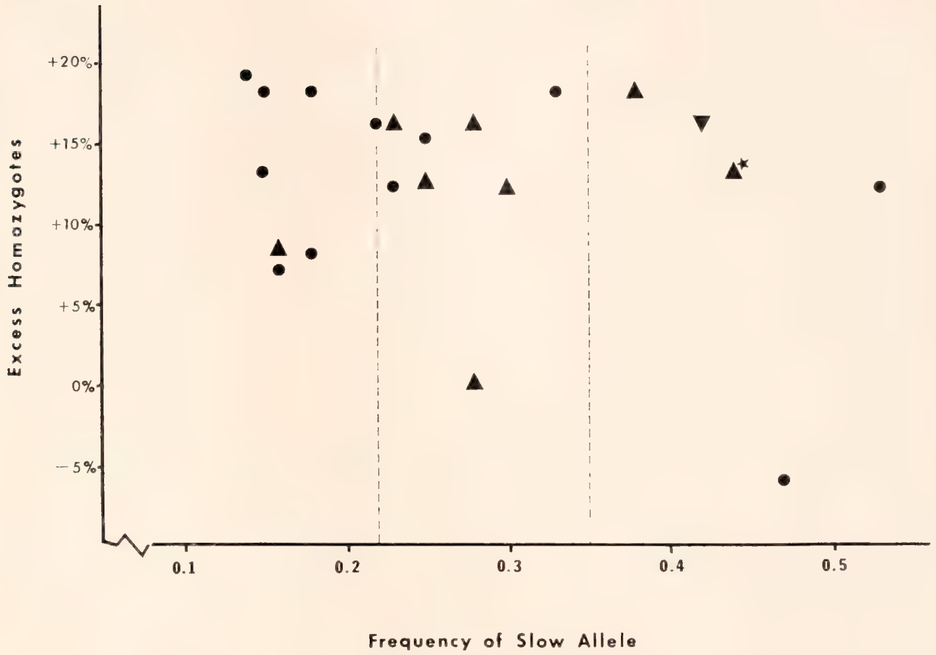

FIGURE 3. Deviations from Hardy-Weinberg expectations of homozygote frequencies *versus* slow frequencies in different size-class samples. Some adjacent size-classes (of Table I) with very similar genotype frequencies have been combined to give larger samples; $N > 65$ for all points except one ($N = 48$) from Scorton Creek (with star). Dotted lines delimit "intermediate" frequencies of slow; Mill Creek/Sandwich Harbor filled circles; Scorton Creek, filled triangles; Sandwich Canal Jetty, filled upside down triangle.

presumably protected them from shifting sand and provided additional anchorage. To summarize, at the Scorton Creek Upstream and Intermediate sites, and at the two Mill Creek sites, and to a lesser extent Stations B and C, mussels were in clumps (several handfuls would fill a bucket), and at the Entrance sites (and Station A) they were collected individually. Although there was discernible water movement either upstream or downstream through the channels throughout most of the tidal cycles, swift roiling currents were observed only in the three hour interval around low tide, where speeds attain five knots. Only the subtidal and low intertidal mussels were subject to the strong currents; presumably only the entrance sites would be exposed to wave action during a storm.

The associated biota seem to have little effect on the mussels themselves. No predators were seen except starfish and oyster drills at the Sandwich Canal Jetty; the small crabs found throughout the marsh were common in the mussel beds. In some samples platyhelminths and nematodes were inadvertently collected—most commonly in the upstream samples and rarely from the sand/rock beaches of the entrance sites. Only at the Barnstable Upstream site was *Modiolus* found with *Mytilus*.

Soft substrate, higher density, larger size, and "upstreamness" generally seem to go together, but no single factor stands out as the major determinant of gene frequency—particularly in view of the fact that an equivalent range of variation for any one factor could probably be found at either Sandwich Jetty (where the mussels were as dense and as large as upstream) or Barnstable.

The localities were studied only over the months of July and August, and it is likely that the greatest ecological contrasts may come in the winter months when productivity declines and freezing and ice impose a severe stress. A length of about 25 mm seems to mark the division of 1st and 2nd year animals (Harger, 1970; Milkman, 1971), and since allelic divergence begins at this size, a seasonal episode might be more important in selection than the differences which result from daily or tidal cycles.

DISCUSSION

The divergence of slow allele frequency with increasing size strongly argues for selection; moreover it suggests a form of balancing selection which would maintain an enzyme polymorphism. Therefore these results parallel and substantiate the selectionist interpretation of Koehn and Mitton (1972) for the LAP locus in *Mytilus* and Koehn, Turano, and Mitton (1973) for the Tetrazolium oxidase (*To*) locus in *Modiolus*. There are however some important differences. First, the *Mytilus* populations described by Koehn and Mitton have a nearly constant S frequency (11%–14%) and the major component of the differences between the sites is the degree of homozygote excess (the statistical tests were made on the genotypes of FF, FX, and XX, where X = M or S). There is no evidence for a clinal pattern at the four sites; whether there is genetic heterogeneity between size classes is not reported.

Modiolus populations at both a high intertidal and low intertidal site have size-dependent changes in Tetrazolium oxidase frequencies (Koehn *et al.*, 1973). These data are pertinent because these sites may be ecological counterparts of the upstream and entrance sites of *Mytilus*, and Koehn and Mitton (1972) have shown that *Modiolus* has a pattern of genic variation similar to *Mytilus* in the Nissequoque River. The TO allele frequencies are essentially constant over size classes and between sites, but at both sites there is an increase with age in the heterozygote relative to the two homozygotes. In *Mytilus* we find—in general—variation in allele frequencies and no trend in Hardy-Weinberg deviations; but for both species, *there is an initial homozygote excess.*

For *Modiolus*, Koehn, Turano, and Mitton (1973) suggest an alternative explanation for this excess, namely selection against heterozygotes at the larval stage prior to settlement. This seems unlikely in the case of the LAP locus in *Mytilus* because there are three heterozygote genotypes which would appear to be equally disadvantaged.

The cause of the persistent Hardy-Weinberg deviations is important in that our conclusions as to *what* is being selected depend upon this issue. If the mussels at each site are immigrants from two (or more) parental populations, then all genes are linked (Milkman and Beaty, 1970) and the LAP locus is a marker of parental origins. Selection is acting in this case on an entire genome, and it is for this reason we anticipate an increasingly better fit to Hardy-Weinberg proportions with age.

If mating is non-random but essentially panmictic, then selection could be acting on the LAP locus and closely linked loci. If the populations are panmictic and all genes are in linkage equilibrium (and the deviations are due to a silent allele), then a strong case could be made for selection at the LAP locus itself.

None of these alternatives is strongly supported by the evidence. The three mussels which produced no stain reaction support the null-allele theory, but they could have resulted from a physiological inactivation rather than a null genotype, and there is the further onus of justifying how this allele could persist in high frequency (around 12%). Inbreeding (or assortative mating) is very plausible for many animal populations, but the gametes of *Mytilus* are spawned into open water. Microgeographic variation, or more likely, temporal variation in spawning correlated with genotype could be responsible for non-random mating. The difficulties with the mixed-population model have already been discussed; of course, to reject it as the explanation for homozygote excess is not to insist that all individuals at a site originate from one population.

As in most studies of this kind, the mussels were collected over a period of time which was brief compared to the life-span of the animal, and the assumption has been made that the pattern we observe is persistent and not due to differential colonization in prior years. R. Milkman has studied a *Mytilus* population in the Cape Cod Canal for three successive years, and reports a consistent colonization pattern over this period. Further, differential colonization as a result of habitat selection would also imply an adaptive role in maintaining the polymorphism.

Although the problem of excess homozygotes has not been resolved, the *Mytilus* data taken as a whole strongly implicate selection as the agent responsible for the pattern of genic variation. The clear divergence of LAP allele frequences with increasing size at two separate, but ecologically similar, localities is good evidence for an adaptive polymorphism in a natural population.

I would like to thank Dr. Roger Milkman for suggesting this study and for his help on numerous occasions. The technical assistance of R. Zeitler and the use of some of the facilities of the Marine Ecology course at Marine Biological Laboratory are gratefully acknowledged. The work was supported in part by the Biological Sciences Development Grant, GM 2591, from the NSF to the University of Iowa and NIH GM 18967 to R. Milkman.

SUMMARY

1. Samples of the mussel, *Mytilus edulis*, were taken from 16 sites on the northern shore of Cape Cod. The mussels were measured and their genotypes at the leucine-amino-peptidase (LAP) locus determined by electrophoresis.

2. At two separate estuarine localities, Sandwich Harbor and Scorton Creek, a pronounced cline in slow allele frequencies in the larger mussels was found.

with upstream sites showing characteristically low frequencies (about 15%) and downstream (entrance) sites having high frequencies (45% to 55%). Mussels smaller than 26 mm had intermediate (22% to 35%) slow frequencies.

3. This clinal divergence with increasing size strongly argues for an adaptive polymorphism. Mechanisms for the observed excess of homozygote genotypes in almost all samples are also discussed.

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FEEDING OF *OVALIPES GUADULPENSIS* (SAUSSURE) (DECAPODA: BRACHYURA: PORTUNIDAE), AND MORPHOLOGICAL ADAPTATIONS TO A BURROWING EXISTENCE

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Ovalipes guadulpensis (Saussure) is a portunid commonly trawled from the sand bottoms along the northwestern Florida Gulf Coast. A sister species, *O. ocellatus* (Herbst), is reported to bury itself in the sand bottoms off North Carolina. When buried the respiratory currents are reversed and the crab emerges from the sand and actively "hunts" for food during rising or falling tides (Pearse, Humm, and Wharton, 1942). The habitats of the two species are similar, except adult *O. guadulpensis* are usually not found near-shore (Williams, 1965).

As no other information was available on the habits of *O. guadulpensis,* a study was undertaken to determine the food and feeding mechanisms of this crab and to assess the morphological adaptations to a burrowing existence.

Materials and Methods

Specimens of *O. guadulpensis* were trawled from sand bottoms in the Gulf of Mexico along northwestern Florida in waters from three to eight meters in depth. Some individuals were preserved upon collection in 70% ethanol and then refrigerated. These crabs were used in stomach content analyses and morphological investigations. Other crabs were kept alive and returned to the laboratory and placed in running seawater aquaria. These were the subjects of observations on feeding, burrowing, and respiratory currents.

Stomach contents and the gastric mill were investigated following the methods of Caine (1974). Motion pictures were made of both feeding and burrowing activity and the film was analyzed frame by frame. Analysis of the film was correlated with the morphology of the appendages and visually verified by repeated observations. Respiratory currents were tested using carmine to follow external currents and small quantities of methylene blue in a seawater solution to trace the internal water pathways.

Observations and Results

Laboratory observations indicated that *O. guadulpensis* was a nocturnally active organism that was buried beneath the sand during periods of daylight. When buried the tips of the antennules may remain exposed, or all parts of the crab may be covered by a layer of sand up to 2 cm deep. When exposed (*i.e.,* active), the crab walked with the dactyli of the walking legs penetrating the substrate and was extremely aggressive with conspecifics or other portunids (aggressive encounters

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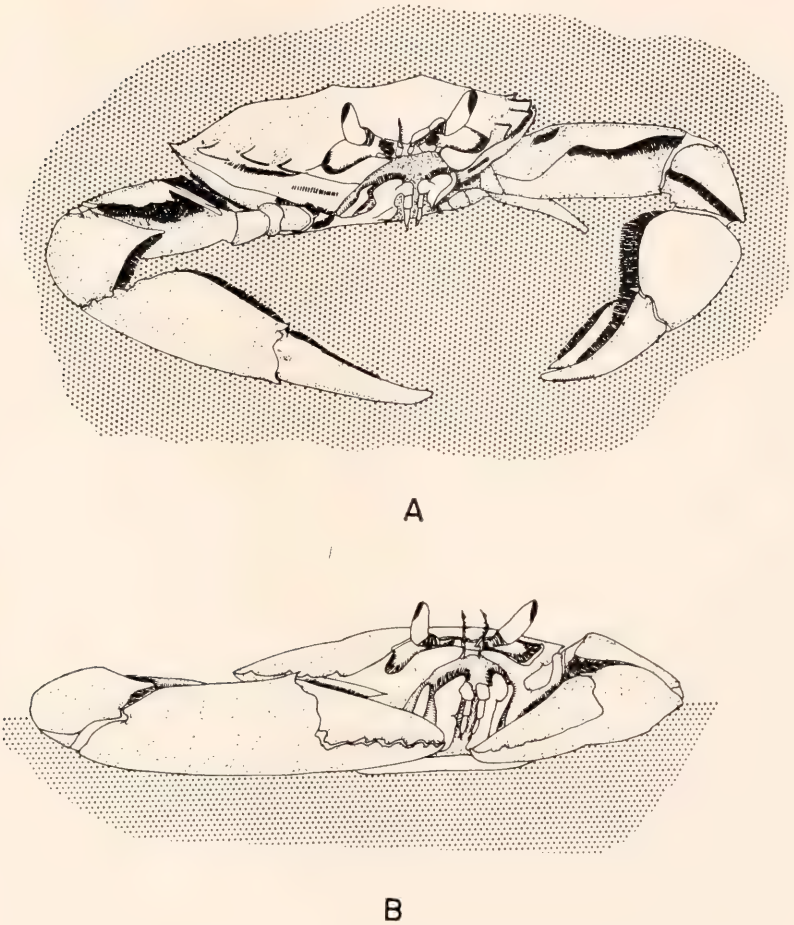

Figure 1. Body postures of *O. guadulpensis,* (A) at rest on the substrate; (B) while burrowing with the chelipeds flexed.

probably do not occur with crabs of other families in nature and no aggressive behavior was directed toward members of other locally occurring brachyuran families when introduced in the laboratory).

Burrowing

When at rest on the substrate, the carapace of the crab was approximately parallel with the surface, the walking legs were at rest on the substrate, and the chelipeds were not tightly flexed against the carapace (Fig. 1A). The substrate was sandy, and the compactness of the sand was thought to dictate the type of burrowing activity. If the bottom was hard packed sand, the crab pushed its left second and fourth walking legs and the right first and third walking legs perpendicularly into the substrate to the level of the distal portion of the merus. The carpus, propodus, and dactylus thus formed an unflexed "probe" into the sand. As the second and fourth left walking legs and the first and third right walking legs were withdrawn from the substrate, the first and third left walking legs and the second and fourth right walking legs were inserted into the sand in a similar manner. The flattened fourth walking legs pushed sand posteriorly as they were withdrawn from the substrate. This procedure was repeated two to four times in rapid sequence (film analyses indicate that a complete cycle for the insertion and withdrawal of the four pair of walking legs took approximately 0.5 seconds). Up to this point the chelipeds and the body of the crab remained relatively stationary.

After the substrate had been sufficiently probed and made less compact (the cessation of probing activity is thought to be related to the proprioceptors in the walking legs and the extent of muscular activity needed to push the legs into the substrate) actual burrowing began. The walking legs continued to move in a manner similar to probing, except the dactylus of each leg was flexed approximately 30° upon full penetration of the substrate, and, instead of withdrawing the appendages, the body of the crab was pulled downward by the same muscular activity. The angle of penetration of the body of the crab in relation to the substrate was about 60°. As the body of the crab began to be buried, the chelipeds were folded tightly against the subhepatic and pterygostomian portions of the body (Fig. 1B). The alternate movements of the legs continued until the chelipeds touched the substrate—the laterally projecting merus, carpus, and propodus forming a barrier.

With all four pair of walking legs extended into the substrate and the dactyli all flexed, the other joints of the walking legs straightened, and the anterior portion of the body of the crab was pushed 0.5–1.0 cm away from the substrate. As the body was elevated, the chelipeds scooped and pushed sand anteriorly from beneath the body of the crab and then returned to a tightly flexed position. The walking legs then contracted in unison and the water between the crab and the substrate was forced from the space, carrying sand with it. As the posterior portion of the crab remained buried and the chelipeds formed an anterior barrier, the main currents of water exit were between the chelipeds and the first pair of walking legs, between the first and second pairs of walking legs, and between the second and third pairs of walking legs. Due to the depression already formed by the burrowing activity, the water exited in an upward direction and, as the waterborne sand settled, the carapace of the crab was covered. The movements also forced the crab deeper into the substrate.

This process was repeated until the crab was buried, with the extent of cheliped movement being an inverse function of the depth of burial. The setose ridges on the chelipeds and along the medio-lateral margins of the pterygostomian and subbranchial regions of the body functioned in blocking water from passing anteriorly, or into the branchial chambers, as the body of the crab was rapidly pulled downwards by the walking legs, thus insuring the movement of water in a lateral and upward direction.

If the substrate was not compressed, the crab probed and dug with the second, third, and fourth legs of one side and then the other side. The mechanics of each leg was the same as previously described for burrowing. The first pair of walking

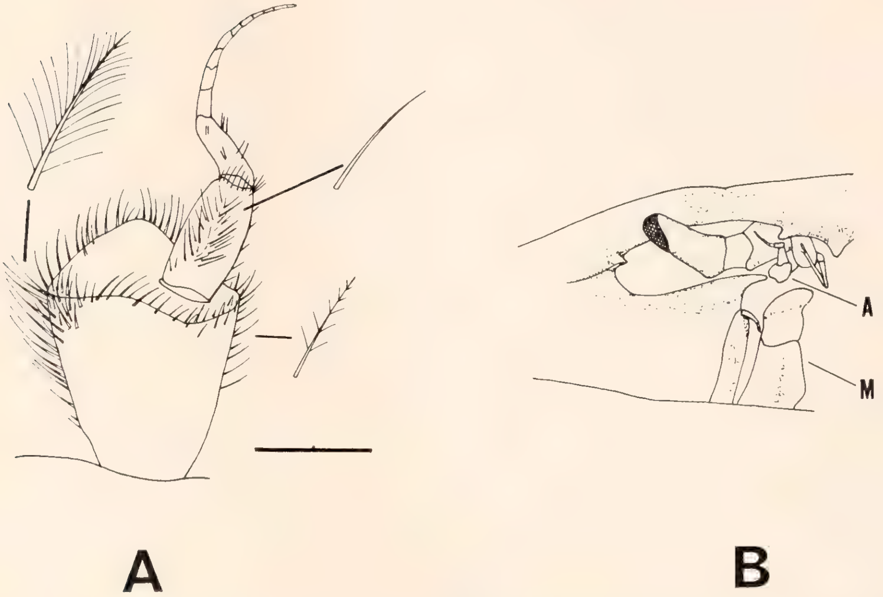

FIGURE 2. Water filtration, (A) second antenna with associated setae, scale bar equals 2 mm; (B) antero-ventral view of the crab showing the placement of the appendages (setae not shown); (A) second antenna; (M) third maxilliped.

legs aided the chelipeds in removing sand from beneath the crab until the chela were partially covered, and then the first pair of walking legs functioned with the respective limbs of the right or left side of the crab. At this point the burrowing mechanisms became the same as for crabs on compressed substrates.

Respiratory pathways

A major problem encountered by burrowing decapods is that of respiration. The gills are encased by the carapace and, in most non-burrowing forms, the respiratory currents pass upwards between the walking legs or through the Milne-Edwards openings dorsal to the basis of the chelipeds and into the branchial chambers. The beat of the scaphognathite pulls water through the branchial chamber and out through the buccal area. Occasionally, short reversed currents occur in non-burrowing forms.

Whether buried or unburied, *Ovalipes guadulpensis* primarily utilized a reversed current with water entering around the scaphognathite, passing through the branchial chamber, and then exhaled antero-lateral to the fifth walking leg. The incorporation of reversed respiratory currents required little morphological modification from the "normal" respiratory pathways of others non-burrowing crabs (described by Borradaile, 1922): the recovery beat of the scaphognathite in normal respiration became the active, pumping beat in reversed currents, and the pumping action during normal flow became the recovery stroke. Water was then pushed,

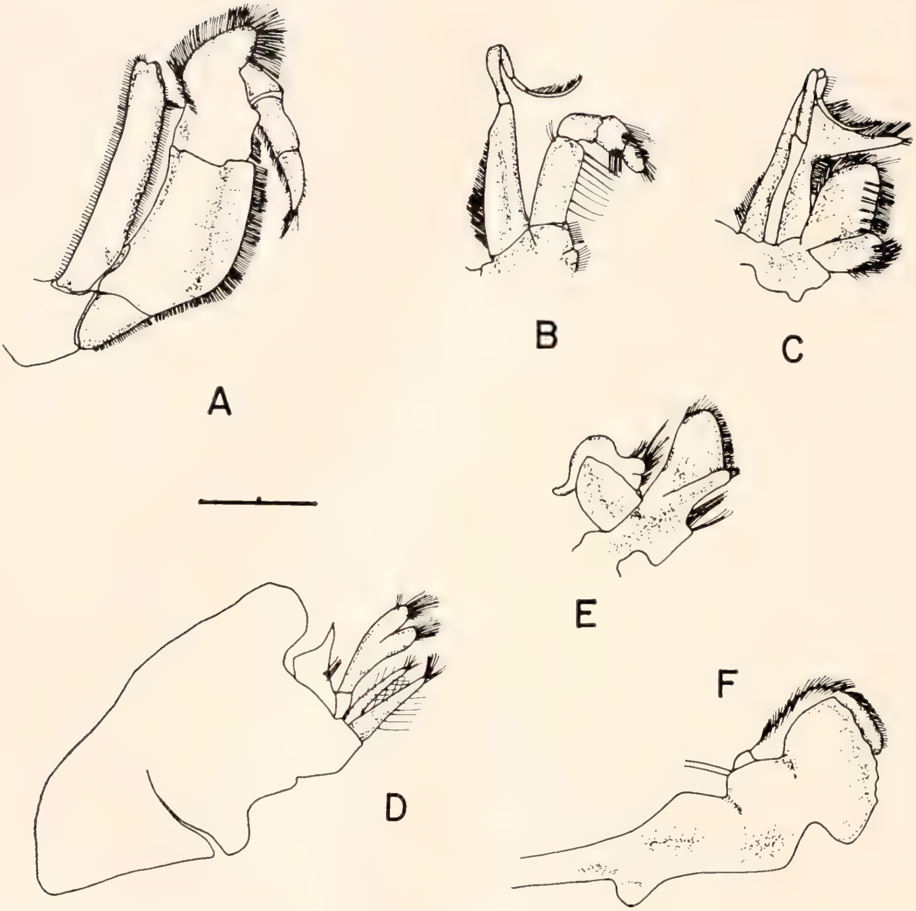

FIGURE 3. Oral appendages of *O. guadulpensis,* (A) third maxillipeds; (B) second maxillipeds; (C) first maxillipeds; (D) second maxilla; (E) first maxilla; (F) manidible. Scale bar equals 2 mm.

rather than pulled, through the branchial regions. Some morphological adaptations occurred in *O. guadulpensis* which helped to insure the exclusion of particulate matter from entering the branchial chambers during reversed water currents. These included: (1) an anterior elongation of the distal portion of the merus of the third maxillipeds (Figs. 1A, 1B, 2B, 3A) which are very setose, (2) the elongation of the distal portion of the endopodite of the first maxillipeds in a medial direction (Fig. 3C) and associated setae on the distal margins, (3) the fusion of the second and third segments of the second antenna, with the fused segments being very setose (Fig. 2A), (4) dense setae around the basis of the walking legs and chelipeds (Fig. 1A), and (5) a reduction in the size and an increase in the capability of closing the Milne-Edwards openings.

During anterior to posterior (reversed) respiratory ventilation, the setae on the distal portion of the first maxillipeds were held against the endostome, with the lateral margins of the appendage also in contact with the endostome. The medial setae of the paired appendages were in close proximity and effectively impeded particulate matter from passing posteriorly. The third maxillipeds occasionally were slightly lowered (*ca.* 5°) to facilitate water entry (the space created by burrowing did not conform with all parts of the crab, a small space filled with interstitial water occurred in the buccal area and near the basis of the fifth pair of walking legs). Most water entered the buccal area around the fused segments of the second antenna (Fig. 2B). The water was thus filtered for sand and detritus at least twice, by the setae on the antennae and then the first maxillipeds, with water occasionally being filtered three times by passing through the setae on the distal portion of the merus of the third maxillipeds after being filtered by the antennal setae.

As the scaphognathite beat posteriorly, the water within the branchial chamber was forced posteriorly with the water being exhaled from the chamber through the posterior openings. As the scaphognathite returned anteriorly, water was drawn through the buccal area and was filtered by the setae on the antenna and first maxillipeds. After passing the first maxillipeds, the water followed the endostome laterally and passed into the branchial chambers through respective openings in the antero-lateral portions of the endostome (one on either side). The openings were created as the anterior portion of the scaphognathite lost contact with the carapace. As the scaphognathite beat posteriorly, these holes were closed.

Water entering the branchial chamber flowed over the dorsal surface of the gills with water flowing in two main pathways, one dorsally and the second being a broad current passing posteriorly and ventro-posteriorly (probably passing between the gill lamellae). The gill lamellae were bathed and cleansed by the water. The maxillipeds were not moving during reversed ventilation, hence the epipodites on these appendages were relatively stationary and not moving along the gills.

Normal respiratory currents (ventral to dorsal and then anterior), which would be reversed for this species, occurred occasionally while buried. When this occurred a stream of water was exhaled vertically from the buccal cavity (the direction of the current due to the third maxillipeds), carrying the sand and detritus from the filtering setae.

Both ventilatory pathways were utilized by non-buried specimens, but reversed currents still predominated. When not buried the chelipeds were not tightly flexed against the body of the crab (Fig. 1A), thus unblocking the Milne-Edwards openings, and the third maxillipeds were lowered ventrally approximately 15°. Water was not filtered as it entered the anterior branchial chambers from both dorsal and ventral openings, but the maxillipeds were often in motion indicating that the epipodites may have been cleansing the gills.

Feeding

Ovalipes guadulpensis was shown to be an opportunistic carnivore on macromaterial by stomach content analyses. Examinations revealed that fish, ophuroids, crustaceans, and annelids composed the majority of the diet, but occasionally molluscs and algae were also found. It appeared that this crab captured unwary prey by rapidly attacking organisms which were detected while the crab was buried. The antennules were seldom buried and often the eyes were also exposed, therefore detection of prey would pose no problem.

The presence of sedentary polychaetes and some molluscs also indicated that some prey were found while the prey, but not the crab, was buried. Detection was probably achieved with the dactyli of the walking legs which contacted the prey organisms while the crab was walking across the substrate. The chemosensitive properties of the dactyli of brachyurans has been documented by Case and Gwilliams (1961) and Case (1964).

Whether the prey was detected while the crab was buried or not, the actual prey capture was accomplished while the crab was not buried. The prey was grasped by one or both of the chela and brought to the oral area. The oral appendages were laterally spread as the prey was forced between the appendages by the chelipeds. All of the oral appendages except the mandibles articulate ventrolaterally and are capable of both medio-lateral and dorso-ventral flexture. The mandibles articulate dorso-laterally and are capable of similar appendage movement (Borradaile, 1922).

The mandibles (Fig. 3F), which were extended ventrally, closed medially on the food material and the third maxillipeds (Fig. 3A), which were extended dorsally, also closed medially on the substance. The mandibles were typical for most decapods in that the molar process was not a masticating surface but more of a serrate cutting edge while the third maxillipeds were typical for most brachyura in that it functioned as an outer covering for the buccal area with the medial margins of the ischium being serrate and forming the *crista dentata.*

As the two pairs of appendages closed medially, the food material was clasped by the molar processes of the mandibles and the *crista dentata* of the third maxillipeds. The mandibles then contracted dorsally as the third maxillipeds contracted ventrally and the food material was usually cut or torn in the region of the mandibles.

The *crista dentata* of *O. guadulpensis* was not exaggerated or strongly serrate, perhaps the function of water filtration with the dense setae on the medial margins of the third maxillipeds dictated a morphological reduction of the *crista dentata.* In any case, the ventral pull of the third maxillipeds were often supplemented by one or both of the chelipeds. The chelipeds, which were already holding the food material, merely pulled antero-ventrally. The food material still broke in the mandibular region.

The endopodites of the second maxillipeds (Fig. 3B) aided in the movement of the nutritive substances through the buccal region while the endites of the first maxillipeds (Fig. 3C) aided in the retention of food materials in close proximity to the mandibles. The two pair of maxilla (Figs. 3D, E) also functioned in the retention of food material, covering the space ventral to the mandibles. The mandibular palp (Fig. 3F) aided in the movement of food into the mouth, as did the endopodites of the second maxillipeds (Fig. 3B), and the labrum (the upper lip).

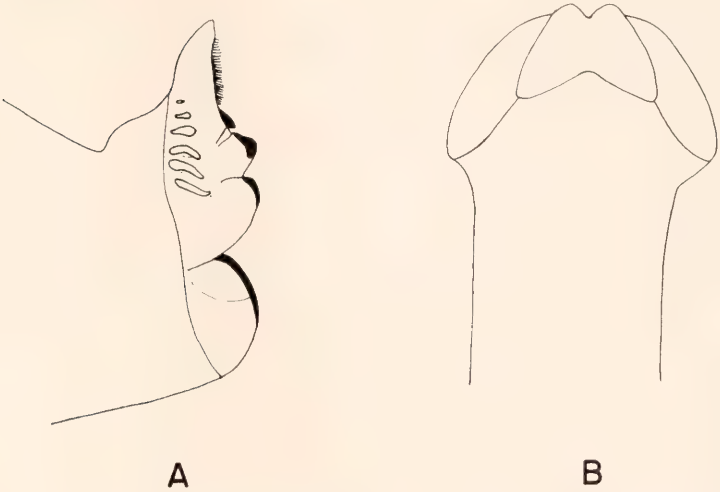

FIGURE 4. Masticating portions of the ossicles of the gastric mill, (A) lateral tooth; (B) median tooth.

Once past the mouth the food passed through the esophagus by peristaltic waves and entered the cardiac portion of the stomach. The food of the Reptantia is masticated and the particles sorted by a collection of setae and ossicles known as the gastric mill (Patwardhan, 1935; Schaefer, 1970). Through muscular contractions, three toothed structures interdigitate and effectively break the food substances into smaller pieces. These three structures, the left lateral tooth (Fig. 4A), the median tooth (Fig. 4B), and the right lateral tooth are interconnected so that upon contraction of certain muscles, the two lateral teeth move medially and the median tooth moves ventrally (Yonge, 1924).

Each lateral tooth (Fig. 4A) was composed of a single, large anterior denticle and a series of three ventro-lateral teeth of progressively decreasing size from posterior to anterior. Dorsal to the ventro-lateral teeth was a series of six vertical ridges. The median tooth complex (Fig. 4B) was composed of a medio-ventral projection which was shaped like a pair of interconnected wings where the broadest portion was directly anteriorly. Lateral to the central projection were two enlarged, elevated ridges. The parts of these teeth that contacted each other were dependant on the extent of muscular contraction.

A strong muscular contraction resulted in the greatest movement of the ossicles so that the anterior denticles of the lateral teeth were touching at the midline with the elevated central projection of the median tooth making simultaneous contact with the anterior denticles. As these structures were the largest and the margins of these structures were the sharpest, it was thought that strong contractions occurred when masticating dense or hard material.

The lateral teeth did not abut when the contraction of the stomach muscles were weaker. There was a space of approximately 0.5 mm separating the lateral teeth so that the lateral ridges of the median tooth passed over the ventro-lateral teeth of each lateral tooth. As the median tooth moved ventrally, the food material was cut by the ventro-lateral teeth and also pressed against and grated by the vertical ridges of the lateral teeth.

Discussion

Ovalipes guadulpensis is a portunid adapted to an existence on hard packed sand, burrowing during periods of daylight and nocturnally becoming active and moving over the substrate. The burrowing mechanisms are advantageous for an existence only on a sand substrate; similar burrowing activity on a coarser substrate such as shell hash or a finer substrate such as silty mud would probably be ineffective. This conclusion is based on the initial probing behavior and the raising and lowering of the body of the crab which creates water currents that aid in burrowing. Experiments to verify the substrate specificity of the burrowing mechanisms were not conducted, though.

Arudpragasm and Naylor (1966) related the predominance of a reversed respiratory current with increased utilization of oxygen from oxygen poor waters. Interstitial water, which forms the bulk of the water circulated over the gills while buried, is probably less saturated with oxygen than open waters.

Morphological adaptations to a burrowing existence are similar to those of *Corystes cassivelaunus* (Pennant) described by Hartnoll (1972), except the antennal flagellae are not setose and do not conduct filtered water to the buccal area. A compact sieve is formed dorsally from the fused second and third segments of the second antenna; ventrally from the merus of the third maxillipeds; and the lateral boundaries are extensions of the pterygostomial region of the carapace. The setose antenna of *Corystes cassivelanus* are probably analogous to the setose distal margin of the first maxillipeds of *O. guadulpensis*.

The range of food ingested by *O. guadulpensis* indicates that the crab is an opportunistic carnivore and scavenger capable of detecting food materials while buried or while actively walking along the substrate. The feeding mechanisms are similar to those described for other carnivorous decapods, but the structure of the lateral teeth of the gastric mill indicate that larger material is more frequently ingested than smaller material.

While this study described various adaptations and habits of *O. guadulpensis*, the factors causing adults to move off-shore were not found.

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BODY FLUID COMPOSITION AND AERIAL OXYGEN CONSUMPTION IN THE FRESHWATER MUSSEL, *LIGUMIA SUBROSTRATA* (SAY): EFFECTS OF DEHYDRATION AND ANOXIC STRESS

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A number of bivalve molluscs are intermittently exposed to air (Bayne, 1973; Kuenzler, 1961; Lent, 1968; Moon and Pritchard, 1970). Usually the valves are closed and it is generally accepted that the animals are under anaerobic conditions (Dugal, 1939; Prosser, 1973). However, some intertidal mussels have been observed to partially gape and consume oxygen from the atmosphere (Coleman, 1973; Kuenzler, 1961; Lent, 1968; Boyden, 1972).

The freshwater mussel, *Ligumia subrostrata,* lives in still water within 50 centimeters of the surface (Murray and Leonard, 1962). These animals will migrate with changes in the water surface level. Occasionally, receding flood waters or drying of the pond leave *L. subrostrata* exposed to the atmosphere. During this time the valves are closed and some animals will survive for several weeks. Other bivalves have survived emersion over 12 months (Dance, 1958).

Epithelial tissues used in gas exchange are permeable to water. When exposed to an atmosphere with high relative humidity, water loss is minimal. However, prolonged exposure to a low relative humidity atmosphere results in significant water loss and concomitant changes in body fluid solute concentrations (Hiscock, 1953).

This report examines the roles of aerial respiration, desiccation and anoxia on changes in body fluid composition and survival of *L. subrostrata* when removed from water. Evidence is presented indicating these animals are not anoxic when out of water but maintain aerobic metabolism. However, they can tolerate forced anoxia.

METHODS

Specimens of *Ligumia subrostrata* were obtained from a pond near Baton Rouge during July 1973 to March 1974. The animal shells were washed with tap water and most were used within one week of collection. Animals kept out of water were maintained at room temperature (22–25° C). Animals not immediately used were stored in tap water (3 mM $NaHCO_3$, 0.1 mM NaCl) or in artificial pond water (Prosser, 1973) at room temperature.

The valves were separated by mechanical force and mantle cavity water drained. The posterior mantle tissue was separated from the shell at the pallial line and reflected anteriorly. The body fluid which accumulates in the exposed pallial space was withdrawn by a pipet. Frequently, the posterior adductor muscle was cut to facilitate fluid collection. The fluid was centrifuged ($8000 \times g$) to remove cells and total solute determined immediately. The remaining fluid was either diluted for other analyses or stored frozen (−20° C).

Fluid analyses

Total solute was determined on undiluted samples of body fluid using a Precision Systems Osmette (0.2 ml) or a Hewlett-Packard vapor pressure osmometer (25 μl). Sodium and potassium were determined on diluted samples using a Coleman flame photometer. Chloride was estimated with a Buchler-Cotlove titrator. Body fluid was diluted with LaO_3-HCl and calcium determined by atomic absorption (Perkin-Elmer).

Lactate and ninhydrin positive substances (NPS) were determined on supernatant of body fluid precipitated with equal volumes of cold 10% trichloroacetic acid and centrifuged at 8000 × *g*. Lactate was estimated by the Ström (1949) modified colorimetric method. NPS was measured by a colorimetric method (Rosen, 1957).

Ammonium excreted by *L. subrostrata* to the bathing medium was measured by direct nesslerization. Equal volumes of bath samples and 10% Nessler's reagent were mixed and nitrogen content determined by colorimetry. Rates of ammonium excretion were estimated from the appearance of nitrogen in the bath.

Tissue glycogen and analyses

Total body weight was determined by weighing the animals after draining the water from the mantle cavity. The soft tissue was dissected from the shell and both shell and tissue were dried to constant weight at 85° C. Shell weight was unchanged when dried further to 105° C, whereas tissue declined by 3.3 ± 0.2% (10).

Total tissue carbohydrates (glycogen) was determined by a phenol-sulfuric acid colorimetric method (Montgomery, 1957). The soft tissue of the mussel was dried to constant weight (85° C) and digested with 20% KOH (100° C). Diluted alkaline digest was analyzed directly for carbohydrate. The alcoholic precipitation of glycogen was eliminated since direct analysis agreed with precipitate analysis (± 4%).

Oxygen consumption

Aerial respiration was determined using a Gilson respirometer. Animals were transferred to 100 ml flasks (Aminco) containing 200 μl water, to maintain water saturated air, and 200 μl 20% KOH as CO_2 absorbant. Oxygen consumption was measured at 22° C for 1.5–3 hr after > 30 min equilibration period. The correction factor for O_2 consumption (Q_{O_2}) was $(P_b \times 273)/(760 \times T)$, where P_b is atmospheric pressure and T is absolute temperature under experimental conditions. All Q_{O_2} values are expressed as μl O_2/g dry tissue-hr at standard temperature-pressure. Sex and season may affect Q_{O_2} (Newell, 1970) but the data are incomplete to attempt corrections.

Oxygen consumption was determined by a modified Winkler method (Strickland and Parsons, 1972). Animals were transferred to containers filled with O_2 saturated pond water and sealed for 1–2 hr at 21° C. At specific times, the containers were opened and water carefully siphoned into 300 ml BOD bottles, with 150 ml overflow, for oxygen determination. The decrease in O_2 content from the O_2 measured initially in the water was used to estimate Q_{O_2}.

Anoxia

Animals were transferred to pond water in a desiccator jar and tubing from a N_2 cylinder connected to an air stone in the water. The nitrogen was continuously flushed through the system and vented to the atmosphere. The pond water was changed daily with pregassed water (<54 μl O_2/l). Animals maintained in a N_2 atmosphere were sealed in a similar container but were supported out of water. The atmosphere was replaced twice each day (5 min flushing with N_2). CO_2 absorbant was also placed in the container.

Data are expressed as the mean ± one standard error. Differences between means were analyzed by the student "t" test and considered significant if $P < 0.05$. Regression lines were estimated by the method of least squares.

RESULTS

Freshwater acclimated animals

Ligumia subrostrata body fluid concentration is among the lowest concentration found in fresh water animals. Tissue and body fluid composition for tap water acclimated mussels is indicated in Table I. Total tissue water has not been partitioned into intra- and extra-cellular compartments. The measured ions account for 72% of the total solute. Bicarbonate is probably a major anion in the body fluid which was not measured (see Potts, 1954).

TABLE I

Tissue and body fluid composition of tap water acclimated L. subrostrata.

Component	Units	Number of animals	Mean ±SEM
Total weight	g	26	23.7 ± 2.4
Shell	g/10g total	26	4.33 ± 0.10
Fresh tissue	g/10g total	26	5.67 ± 0.10
Mantle cavity water	g/10g total	10	1.2 ± 0.1
Tissue water	g/10g fresh tissue	18	8.8 ± 0.0
Body fluid:			
Total solute	mOsmoles/l	26	53.0 ± 0.6
Na	mM/l	26	21.0 ± 0.6
Ca	mM/l	8	4.8 ± 0.2
K	mM/l	10	0.4 ± 0.0
Cl	mM/l	26	12.0 ± 0.6
Lactate	mM/l	15	0.14 ± 0.01
NPS*	mM/l	20	1.28 ± 0.17

* Ninhydrin positive substance.

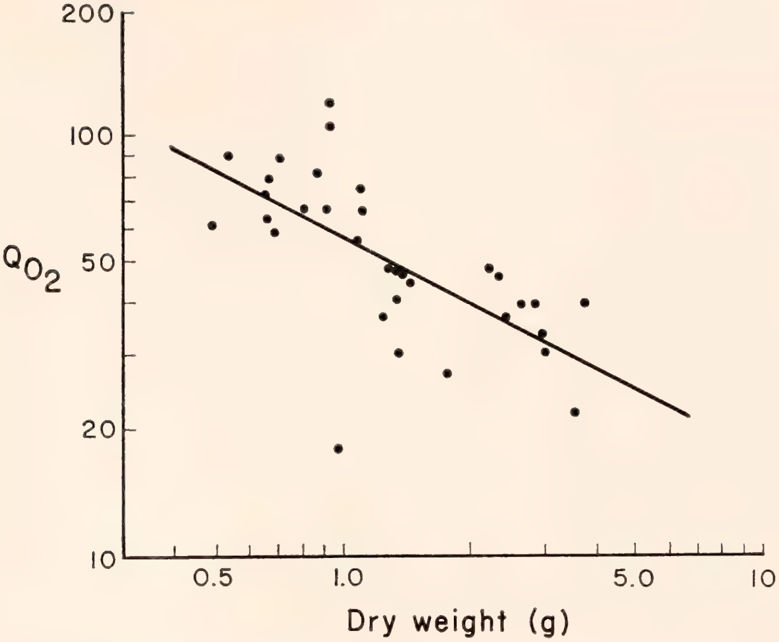

FIGURE 1. Aerial oxygen consumption in *L. subrostrata* as a function of dry tissue weight. The Q_{O_2} is in μl O_2/g dry tissue-hr. The slope of the regression line is − 0.51 ± 0.02 (35).

Freshly collected animals excrete ammonia. The animals were placed in individual containers with tap water and aliquots taken for nitrogen determination. The average net flux of ammonium was 1.1 ± 0.1 μm N/g dry tissue-hr (15) over a one hr interval.

Oxygen consumption

L. subrostrata transferred from water to a respirometer flash consumes oxygen from the air. The weight specific Q_{O_2} is a logarithmic function of dry tissue weight (Fig 1). Although the weight range is narrow, the correlation ($r = -0.68$) is

TABLE II

L. subrostrata *oxygen consumption from water saturated air. Values are listed as mean ± 1 SEM. A, in laboratory tap water less than 5 days; B, in laboratory tap water 12 weeks; C, in sealed container with water saturated air for 30 days.*

Condition	N	Tissue dry weight, g	Q_{O_2}
A	20	1.38 ± 0.21	59 ± 6
B	5	1.40 ± 0.11	42 ± 6
C	10	1.52 ± 0.15	80 ± 8*

* $P < 0.05$.

TABLE III

L. subrostrata *oxygen consumption from air saturated pond water. Values are listed as mean ± 1 SEM. A, in laboratory pond water less than 5 days; B, in laboratory pond water 12 weeks.*

Condition	N	Tissue dry weight, g	Q_{O_2}
A	10	2.16 ± 0.30	287 ± 16
B	7	1.29 ± 0.06	185 ± 17*

* $P < 0.001$.

highly significant ($P < 0.001$). The aerial Q_{O_2} is not significantly changed after several months laboratory storage in tap water (Table II). Variability in Q_{O_2} was high, ranging from 18 to 150.

Oxygen consumption in water is significantly higher than aerial respiration (Table III). The Q_{O_2} from mussels stored in tap water several months was significantly (36%) less than recently collected animals, however, the difference in weight tends to minimize the reduction. Aquatic respiration exceeds aerial Q_{O_2} even after prolonged storage in tap water. Part of the elevated Q_{O_2} in water is due to activity; siphoning water, valve and foot movement. There was no change in Q_{O_2} when the stored animals were pretreated 24 hr with penicillin (800 u/ml) in the water: Q_{O_2} 160 ± 37 (5).

The weight specific aquatic Q_{O_2} is a logarithmic function of dry tissue weight (Fig. 2). The slope of the regression line is significantly different from that noted for aerial respiration ($P < 0.001$). All animals used for the regression analysis

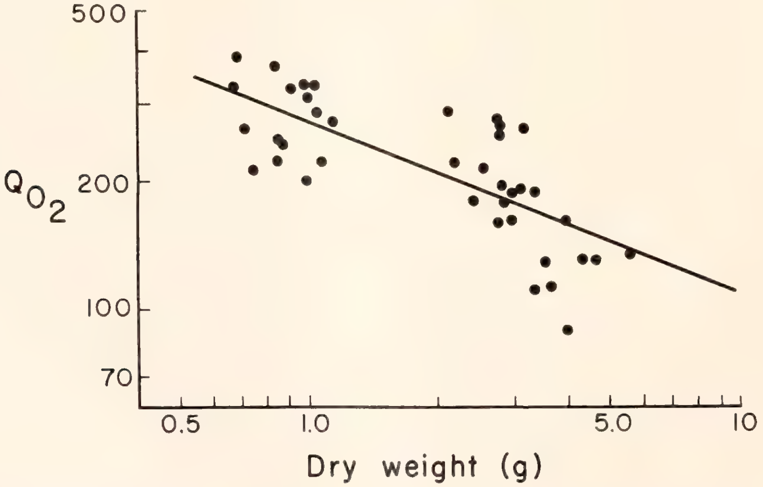

FIGURE 2. Aquatic oxygen consumption in *L. subrostrata* as a function of dry tissue weight. The Q_{O_2} is in μl O_2/g dry tissue-hr. The slope of the regression line is -0.40 ± 0.06 (39) and the correlation is highly significant ($r = -0.75$, $P > 0.001$).

were stored in pond water less 7 days. By 14 days a detectable drop of Q_{O_2} was noted which becomes pronounced with long term storage (*cf.* Table III).

Effects of prolonged exposure to air

Animals transferred to a sealed container, with water saturated air and CO_2 absorbant, moved intermittently during the first few hours. After 12 hr, movement ceased and the valves appeared closed. The animals continued to consume O_2 from the atmosphere even after 30 days exposure. The Q_{O_2} was higher than values observed for animals out of water less than 3 hr, however, part of the increase may be due to microbe contamination, which was not controlled. These animals lost 1.44 ± 0.16 g/10 g fresh tissue weight over the 30 day interval. However, the body fluid composition was similar to that found in animals subjected to 2 days dehydration at lower humidity (see below).

Total tissue glycogen in freshly collected mussels was 0.36 ± 0.02 g/g dry tissue (10). Tissue carbohydrate was unchanged after the animals were exposed to air (high humidity) for 40 days (36 ± 1% of dry weight, N = 10). This is 88–95% of tissue glycogen from fresh animals at that season.

Survival of *L. subrostrata* is reduced to 6–10 days if they are exposed to a low relative humidity atmosphere (45–55%) at 25° C. Oxygen consumption from air is significantly increased after 4–5 days dehydration: Q_{O_2} 100 ± 24 (7). Microbial contamination may contribute to the elevated Q_{O_2}. Particulate matter accumulates in the mantle cavity near the siphons when dehydrated. These animals lost 2.40 ± 0.68 g/10 g fresh tissue weight.

During dehydration, the rate of evaporative water loss is relatively constant over a 4 day period (30 μl/10 g fresh tissue-hr). The loss of water results in an increased body fluid total solute concentration (Table IV). The highest total solute measured was 160 mOsmoles/l. The initial changes in calcium are higher than expected and may be due to calcium dissolved from the shell to buffer accumulation of metabolic products. Both lactate and NPS are elevated relative to

TABLE IV

Effect of dehydration on body fluid ion composition in L. subrostrata. *Data are expressed as mean ± 1 SEM, number of animals in parenthesis. Animals were maintained in air at 25° C, 45 to 55% relative humidity.*

Component	Units	Days of dehydration		
		2	4	7
Total solute	mOsm/l	68.8 ± 1.7 (8)	89.0 ± 2.7 (12)	91.9 ± 7.8 (14)
Na	mM/l	24.5 ± 0.8 (8)	32.9 ± 0.6 (12)	37.5 ± 1.2 (14)
Cl	mM/l	18.4 ± 0.6 (8)	29.4 ± 1.7 (12)	35.2 ± 2.0 (14)
Ca	mM/l	8.4 ± 0.6 (8)	8.0 ± 0.9 (9)	—
Lactate	mM/l	0.3 ± 0.1 (8)	0.8 ± 0.2 (7)	0.8 ± 0.2 (7)
NPS	mM/l	3.6 ± 0.1 (8)	2.8 ± 0.2 (12)	3.8 ± 0.5 (7)
Total H_2O loss	g/10g*	1.46 ± 0.22 (8)	2.85 ± 0.03 (8)	3.44 ± 0.14 (7)

* Based on initial fresh tissue weight.

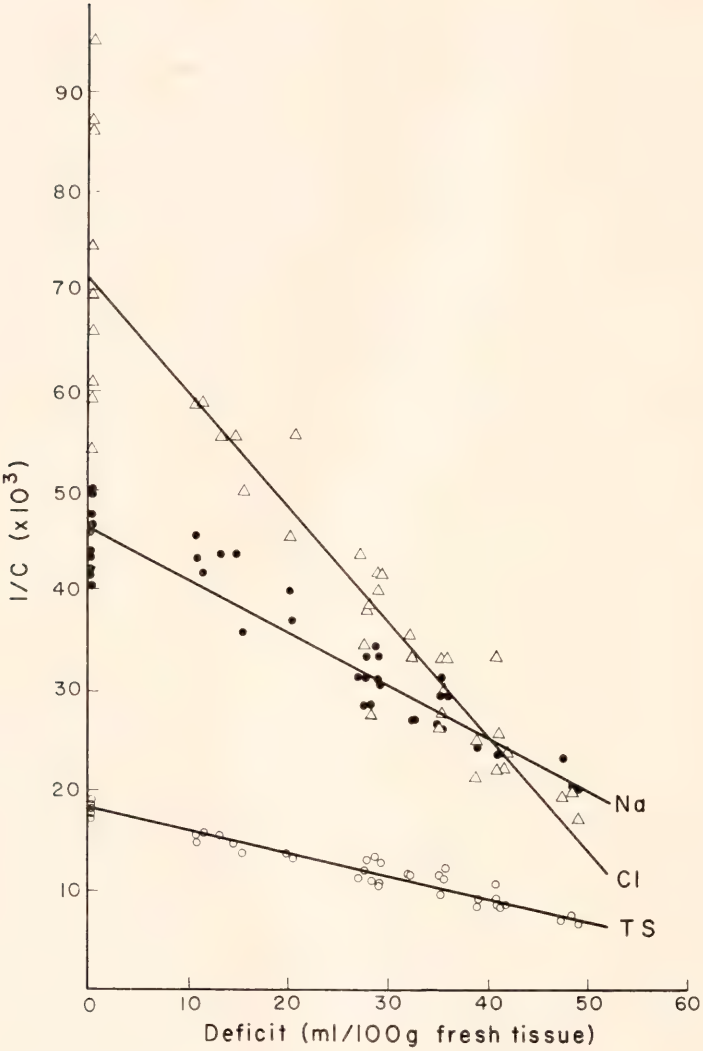

FIGURE 3. The effect of dehydration on solute concentration in the body fluids of *L. subrostrata*. The abscissa is the water lost during dehydration. The deficit is based on initial fresh tissue weight excluding the shell and mantle cavity water. The ordinate is the reciprocal of solute concentration $(\text{mM}/1)^{-1}$ multiplied by 10^3. TS is the total solute $(\text{mOsm}/1)^{-1}$. Number of animals used for determining the regression lines was 46.

tap water controls. However, no major changes were noted with prolonged dehydration.

It is possible to relate the body fluid solute concentration to the degree of dehydration in an animal (Alvarado, 1972). Assuming the water in a compartment is available as solvent and, if there are no changes in the solute content in the compartment, then it is possible to predict the body fluid concentration as a function of water deficit:

$$C = (C_o \cdot V_o)/(V_o - D) \tag{1}$$

Where C_o and V_o are the solute concentration in the body fluids and total water content of a hydrated animal (excluding the shell), respectively; C is the body fluid solute concentration after dehydration and D is the water deficit. The reciprocal of Equation 1 can be rearranged into an equation for a straight line:

$$1/C = 1/C_o - D/(C_o \cdot V_o) \tag{2}$$

Figure 3 shows the data plotted using Equation 2. The correlation between water deficit and Cl, Na or total solute is highly significant ($P < 0.001$) with correlation coefficients (r) for each regression line: −0.90, −0.95 and −0.97, respectively. Linear regression estimates of the Y-intercept ($1/C_o$) and the X-intercept (V_o) are presented in Table V. The calculated and observed C_o values are in agreement. However, the V_o estimates from Cl and total solute are significantly different from the observed values. These data indicate a shift of Cl out of tissues into the body fluid.

Effects of anoxia

Exposure of *L. subrostrata* to water saturated N_2 atmosphere resulted in a significant behavioral change. Within 12 hr of exposure to anoxic conditions, the valves gaped and the foot was extended several centimeters. This is apparently an attempt to expose additional tissue surface area for oxygen absorption. Living animals responded to repeated mechanical stimulation by slowly withdrawing the foot but re-extend it within a few hr. These animals could survive 4–6 days anoxia. The magnitude of oxygen debt was noted when 7 animals were returned to air in a respirometer flask: Q_{O_2} 249 ± 56 over a 1 hr interval. Total tissue glycogen was not changed from the tap water acclimated animals.

Exposure of *L. subrostrata* to a N_2 atmosphere resulted in significant changes in the body fluid composition (Table VI). Total solute increased rapidly even though dehydration was minimized. The maximum total solute measured in a

TABLE V

Calculated and observed values of solute concentration and water content of L. subrostrata. *All values are mean ± 1 SEM, number of animals in parentheses.*

Solute	C_o(mM/l)		V_o(ml/100 g tissue)	
	Calculated*	Observed	Calculated*	Observed
Na	21.7	21.0 ± 0.6 (26)	89.1	87.7 ± 0.4 (18)
Cl	14.0	12.0 ± 0.6 (26)	62.6**	87.7 ± 0.4 (18)
Total solute	54.4	53.0 ± 0.6 (26)	79.9**	87.7 ± 0.4 (18)

* From least squares regression estimates, n = 46.
** $P < 0.001$.

TABLE VI

Effect of N_2 atmosphere on body fluid ion composition in L. subrostrata. *Data are presented as mean ± 1 SEM, number of animals in parentheses. Animals were in sealed containers at 25° C, 100% R.H. gassed with N_2 twice daily.*

Component	Units	Days of anoxia	
		2	4
Total solute	mOsm/l	107 ± 7 (14)	121 ± 6 (20)
Na	mM/l	20.0 ± 0.6 (14)	19.9 ± 0.6 (20)
Cl	mM/l	12.9 ± 0.5 (14)	11.4 ± 0.6 (20)
Ca	mM/l	26.3 ± 4.0 (14)	28.0 ± 3.4 (6)
Lactate	mM/l	6.3 ± 0.9 (14)	6.7 ± 1.4 (10)
NPS	mM/l	4.1 ± 0.4 (8)	4.2 ± 0.4 (4)
Weight loss	g/10g*	0.65 ± 0.15 (8)	0.69 ± 0.17 (10)

* Based on initial total animal weight.

surviving animal was 180 mOsmoles. Sodium and chloride do not change relative to tap water controls. The major ionic change is a 5 to 8 fold increase in calcium and the unidentified anion (see discussion). The highest measured calcium concentration was 41 mM/l. The body fluid lactate concentration also increased 50 fold relative to tap water animals but the contribution to total solute is small. The NPS increased above tap water animals but the chemical nature is not known. The weight loss may be due to dissolved shell and subsequent evolution of CO_2 rather than water loss since the Na and Cl concentrations were not changed.

Although few animals survive longer than 4–6 days in a N_2 atmosphere, survival is extended if the animals are immersed in N_2 gassed water (> 15 days). The animals did not extend the foot but all siphoned water. Apparently these animals do not accumulate an oxygen debt under these conditions. When returned to air the Q_{O_2} was 73.0 ± 14.6 (5) over a 1 hr interval.

The body fluid composition is modified when exposed to N_2 gassed pond water (Table VII). The body fluid total solute is significantly higher than animals in aerated tap water but less than animals in N_2 atmosphere. Calcium and lactate

TABLE VII

Effect of exposure to N_2 gassed water on body fluid ion composition in L. subrostrata. *Data are presented as mean ± 1 SEM.*

Component	Units	Days exposure*	
		4	15
Total solute	mOsm/l	63 ± 2	68 ± 1
Na	mM/l	15.4 ± 0.5	12.4 ± 0.4
Cl	mM/l	6.0 ± 0.3	4.1 ± 0.2
Ca	mM/l	14.4 ± 1.0	10.9 ± 0.6
Lactate	mM/l	0.26 ± 0.05	0.23 ± 0.03

* N = 6 animals for each experiment.

are elevated in response to anoxia but excess metabolic acids are apparently excreted. The measured net loss of calcium to the bathing medium was 2.0 ± 0.1 μm Ca/10g fresh tissue-hr over 15 days exposure to N_2 gassed water. Metabolic acid excretion was not monitored because of potential decomposition by microbes in the water. The reduction in body fluid sodium and chloride is significant but has not been pursued.

Discussion

If water loss is retarded, *L. subrostrata* will survive over 40 days in air and other pelecypods exceed three weeks (Bayne, 1973; Dugal, 1939). Dance (1958) has reported a freshwater mussel survived 12 months out of water. Those animals which have been studied were not anoxic but consumed O_2 from the atmosphere (Coleman, 1973; Kuenzler, 1961; Lent, 1968). The limiting factor for survival in air is primarily tolerance to desiccation. Although the body fluids of freshwater mussels are dilute (Potts, 1954), *L. subrostrata* will tolerate a three fold increase in body fluid total solute, with an upper limit of 160 mOsmoles. Analyses of the body fluids indicate the higher osmolarity is due to water loss and concentration of solutes rather than a build up of metabolic products.

It is noteworthy that these animals tolerate extensive changes in body fluid total solute and qualitative changes in ionic composition. They are able to survive a large increase in NaCl concentration during dehydration and an 8 fold increase in body fluid Ca during anoxia. The shift of Cl from the tissues to the body fluid during dehydration and the reduction of body fluid Na and Cl during anoxia in water are significant but the mechanism remains obscure. Apparently, the osmolarity of body fluids is the primary factor limiting survival and the specific solutes are less important.

A source of energy for survival out of water is glycogen (Stokes and Awapara 1968; De Zwaan and Zandee, 1972b). The quantity of total carbohydrate (36% of dry tissue weight) in *L. subrostrata* is similar to other pelecypods (Badman and Chin, 1973; De Zwaan and Zandee, 1972a, 1972b). Potential survival time, in air, for an animal of one gram dry tissue weight, using glycogen only, is about 200 days. Aerobic metabolism will also allow protein and lipid catabolism. Curiously, others have noted more rapid tissue glycogen loss (Badman and Chin, 1973; De Zwaan and Zandee, 1972b). In water, *L. subrostrata* excretes ammonia but out of water the nitrogen from protein catabolism may shift into less toxic urea production (Andrews and Reid, 1972). This may contribute to the elevated NPS production noted during dehydration.

When pelecypods are returned to water, oxygen consumption is higher with a Q_{O_2} about 3–4 times the aerial rate (Coleman, 1973; Kuenzler, 1961; Moon and Pritchard, 1970). This is probably reflecting the increased energy required in moving water to irrigate the mantle cavity. Animals recently collected have a greater Q_{O_2} in water than animals stored in the laboratory for a period of time. Which value is "normal" cannot be established by these experiments. The higher Q_{O_2} may be due to greater foot movements, especially prominent during the first 2 weeks, or ventilation "searching" for food. Animals under both conditions have a high glycogen content so depletion of food stores is not the primary reason for low Q_{O_2} in laboratory adapted *L. subrostrata*. However, it has been suggested that starvation is the reason for the lower Q_{O_2} in some gastropods which have a lower carbohydrate reserve (von Brand, Baerstein and Mehlman, 1950). A transient higher Q_{O_2} immediately after reimmersion in water (Moon and Pritchard, 1970) may be due to some oxygen debt or the energy required to remove trapped air and wastes accumulated in the mantle cavity during exposure to air (Boyden, 1972). Specimens of *L. subrostrata* were observed to open the valves allowing air to escape from ventral and posterior shell margins followed by forceful adduction of the shell which expelled additional air and particulate matter from the siphon area.

L. subrostrata is a facultative anaerobe. However, when exposed to anoxic conditions out of water, survival time is not longer than animals exposed to dehydration in air (5–7 days). The body fluid total solute is elevated after prolonged exposure to N_2 but the maximum concentration tolerated is 180 mOsm. This is about the same for animals exposed to dehydration. However, the principal solutes contributing to the osmolarity in anoxic animals are calcium and its anion; probably bicarbonate and succinate (Dugal, 1939; Potts, 1954; Stokes and Awapara, 1968; De Zwaan and Zandee, 1972a, 1972b).

The ability of *L. subrostrata* to tolerate prolonged anoxia in water (>15 days) is of ecologic significance. It is not unusual for water with a high organic content to drop to near zero oxygen for extended periods. Some gastropods will also tolerate anoxia in excess of two days, although there are considerable species differences (von Brand, *et al.*, 1950).

The metabolic adaptations to facultative anaerobiosis in bivalves include production of the less toxic alanine and the less soluble succinate salts rather than lactate (Stokes and Awapara, 1968; De Zwaan and Zandee, 1972b). Recently, Hochachka and Mustafa (1973) have demonstrated that anaerobic metabolism in the Pacific oyster is adapted to generate additional substrate level phosphorylation as α-ketoglutarate is shunted into the Krebs cycle (Mustafa and Hochachka, 1973). The ability to excrete metabolic acids to the environment is energetically expensive but prevents the build up of body fluid osmolarity which would be lethal.

I wish to thank Ms. Gerry Bullock and Christine Angelloz for typing the manuscript, E. Stern for identifying the animals and M. Nowakowski for technical assistance. This work was supported, in part, by the Louisiana State University Graduate School Research Council.

SUMMARY

1. *Ligumia subrostrata*, removed from water will survive >40 days if dehydration is minimized by high relative humidity. They are not anoxic but consume oxygen from the air (Q_{O_2} 59 μl O_2/g dry tissue-hr). One source of energy is from the large glycogen stores (36% of dry tissue).

2. Animals removed from water and exposed to either low relative humidity (45–55%) or N_2 atmosphere will survive about 5–7 days. The maximum total solute in the body fluids of surviving animals is 160–180 mOsmoles.

3. Sodium, Cl, K, and Ca account for 72% of the total solute in the body fluids of fresh water acclimated animals. Dehydration increases the concentration of Na proportional to the amount of water lost. There is a significant shift of Cl from the tissues into the body fluids during dehydration.

4. When the animals are out of water, forced anoxia increases the body fluid total solute but the Na and Cl contribution is small. The major ions in the body fluids are calcium and bicarbonate or succinate; reflecting a build up of metabolic acids. Lactate concentration in the body fluids is low. These animals experience a significant oxygen debt since oxygen consumption is elevated when returned to air (Q_{O_2} 249 μl O_2/g dry tissue-hr).

5. Mussels are facultative anaerobes and will survive for extended periods of time in N_2 gassed water (< 54 μl O_2/1). The body fluids are slightly changed from normal suggesting the metabolic products are excreted. These animals do not suffer an oxygen debt.

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LARVAL SETTLEMENT OF A SYMBIOTIC HYDROID: SPECIFICITY AND NEMATOCYST RESPONSES IN PLANULAE OF *PROBOSCIDACTYLA FLAVICIRRATA*

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Planktonic larvae of many benthic invertebrates preferentially settle and metamorphose on specific substrates (Williams, 1964; Newell, 1970). Frequently, the planktonic larvae of symbionts possess exceptionally well-developed physiological mechanisms for substrate selection. Such mechanisms presumably evolved because, for these species, only a small proportion of the surfaces that a larva might contact are suitable for settlement (Davenport, 1955). I have studied planulae of the symbiotic hydroid *Proboscidactyla flavicirrata* (Brant). The structure of a planula larva is extremely simple, yet the planulae of *Proboscidactyla* settle with great specificity (Campbell, 1968).

Sabellid polychaetes are the only known hosts of *Proboscidactyla* (Gosse, 1857; Uchida and Okuda, 1941; Hand, 1954; Brinkmann and Vannucci, 1965; Calder, 1970). *Proboscidactyla* colonies are found on the rims of sabellid tubes where their gastrozooids can contact the host's plume of tentacular cirri. According to Campbell (1968), *Proboscidactyla* planulae begin settlement by adhering with nematocysts to the pinnules that branch from the tentacular cirri. Later, when the sabellid retracts its tentacular cirri into its tube, the planulae somehow transfer to the rim of the tube where they metamorphose.

In this report I confirm and extend Campbell's work. Two new findings are of particular importance. First, planulae use nematocysts both to attach to pinnules as Campbell (1968) suggested and to transfer to the tube rim. Between the time of attachment to a pinnule and transfer to the tube rim, the effective stimulus for nematocyst discharge shifts from pinnule to worm tube. Secondly, planulae settle and metamorphose on either of two sympatric species of sabellids, one of which does not support *Proboscidactyla* colonies.

MATERIALS AND METHODS

During June, July, and August of 1973, the medusae of *Proboscidactyla* were collected from surface waters, and the sabellids *Schizobranchia insignis* and *Eudistylia vancouveri* from the undersides of mooring floats near the University of Washington Friday Harbor Laboratories. Nearly mature *Proboscidatyla* medusae sometimes ripened when fed *Artemia* larvae. The medusae, embryos, and planulae were kept in glass finger bowls or Syracuse dishes partially submerged in an open-circulation sea table and the water in the holding containers was changed at least every third day.

The medusae shed their gametes at night and developing embryos were routinely transferred to Syracuse dishes the next morning. Zygotes were sometimes collected during the night to examine early development. Individual planulae, severed sabellid pinnules, and fragments of sabellid tubes were handled using tygon suction micropipettes (Josephson, 1965) held in micromanipulators.

The micrographs illustrating this report were made with incident illumination using either a Wild M-5 stereo microscope, or a Zeiss photomicroscope equipped with Nomarski differential interference contrast optics.

RESULTS

Development

Cleavage in *Proboscidactyla* eggs was holoblastic, unilateral, and equal or nearly equal. Cleavages 2 through 7 occurred at approximately 45 min intervals (14° C). A blastula had formed by 6 hr after fertilization. After about 10 hr individual blastomeres began to migrate inward to form the entoderm. In the majority of the embryos this migratory activity was basically unipolar, although small groups of cells sometimes ingressed away from the main site of gastrulation. In a few embryos, migratory cells were distributed almost uniformly over the inner surfaces of gastrulae. Nevertheless, as gastrulation progressed, the entoderm always thickened toward the pole that eventually became the posterior end of the swimming planula (see Fig. 1).

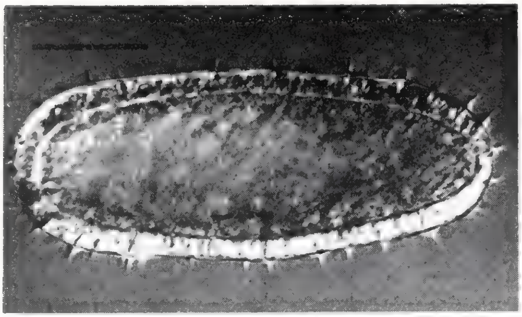
Cilia first appeared 14 hr after fertilization and at about 16 hr the planulae began to crawl and swim. At approximately the same time the planulae started to change shape, alternately becoming ovoid and nearly spherical. Planulae swam and crawled only when in the ovoid shape. Those crawling along the bottoms of dishes advanced without rotating, but swimming planulae always rotated slowly on their long axes in a clockwise direction when viewed from the anterior end. According to Widerstein (1968) such clockwise rotation is a common feature in cnidarian larvae.

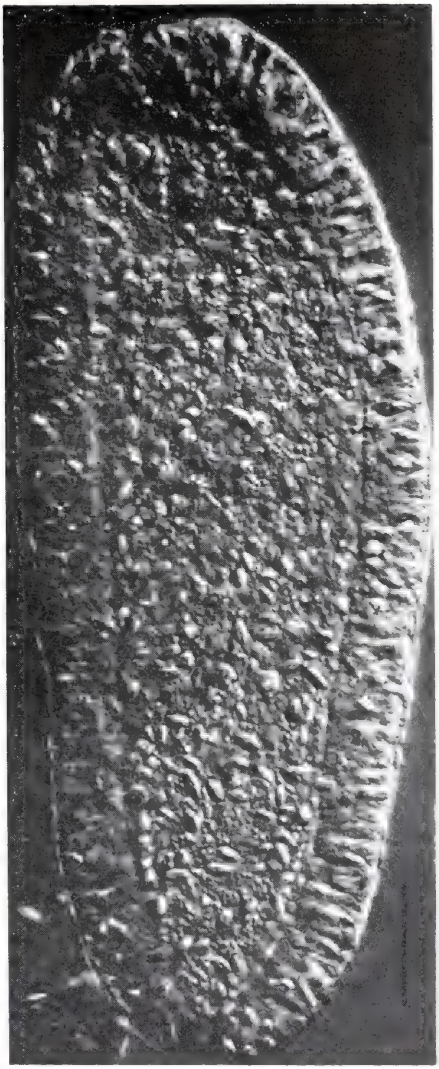
The Cnidome

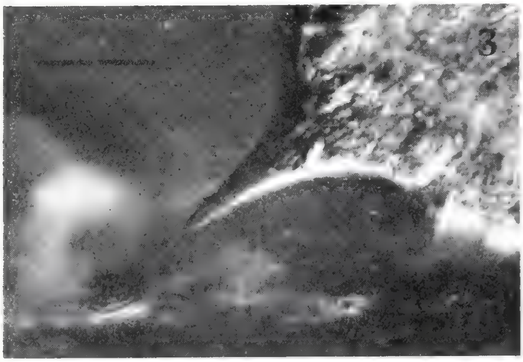
Cnidocils began to protrude from the epidermis about 48 hr after fertilization. During the 24 hr that followed, their numbers gradually increased, presumably as more and more nematocysts matured or reached peripheral locations. During this period of development the planulae were periodically touched with severed *Schizobranchia* pinnules to determine whether they had acquired the ability to attach.

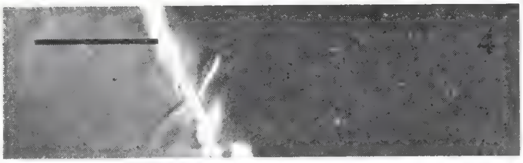
Prior to the appearance of cnidocils about 36 hr after fertilization, planulae never adhered to pinnules even momentarily. About 48 hr after fertilization, while the cnidocils were still comparatively sparse, planulae sometimes adhered but failed to remain attached for more than a few seconds. After 72 hr the density of cnidocils on the surface appeared to have reached its maximum. Planulae that touched *Schizobranchia* pinnules more than 72 hr after fertilization always attached and usually remained attached even if jostled vigorously.

Peripheral nematocysts in fully developed planulae were distributed almost uniformly over the ectoderm (Fig. 1). No concentration of nematocysts in the ectoderm of the anterior end comparable to that reported by Campbell (1968) was









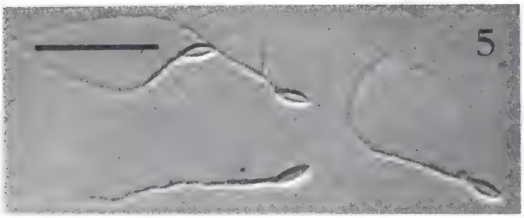


FIGURE 1. Protruding cnidocils distributed fairly uniformly in the epidermis of a fully developed planula. The cavity within the planula appears as a lighter area centered nearer the anterior end (left). The entoderm is correspondingly thicker toward the posterior end; scale bar = 50 μ.

FIGURE 2. Optical section of a planula showing a concentration of nematocyst capsules in the posterior entoderm (bottom third). (Fig. 2–5 are all to the same scale and the nematocyst capsules depicted in these figures all appear similar); scale bar = 25 μ.

FIGURE 3. Epidermal distortion formed by pulling an adhering planula away from a fragment of sabellid tube; scale bar = 25 μ.

FIGURE 4. Nematocyst discharged into a fragment of sabellid tube. A portion of the embedded thread can be seen leading down and left from the capsule; scale bar = 25 μ.

FIGURE 5. Discharge epidermal nematocysts; scale bar = 25 μ.

apparent. Nematocyst capsules were present in the entoderm as well, mainly toward the posterior end where the entoderm was thickest (Fig. 2).

Mechanism of adhesion

In nature, a *Proboscidactyla* planula contacts 2 different substrates during the course of settlement. Initially it adheres to a sabellid pinnule (attachment). While still attached to the pinnule it adheres to the rim of the sabellid's tube (the initial event in transfer). The simultaneous acquisition of cnidocils and attachment capability suggested that nematocysts are responsible for adhesion to pinnules. This was confirmed by pulling attached planulae off pinnules with micromanipulators. As planula and pinnule separated, areas of the planula epidermis stretched into cones attached to the pinnule at their apices (Fig. 3). At each apex was a discharged nematocyst with its thread embedded in the pinnule. Similar experiments performed with planulae that had transferred from pinnules to fragments of sabellid tube produced identical results. Nematocyst capsules left upon pinnules and tube fragments were also observed after planulae had been dislodged from their sites of adhesion. If the site was the edge of a thin piece of tube the embedded thread could sometimes be seen as well as the capsule (Fig. 4). Although each planula possessed over a hundred epidermal nematocysts, only a few (usually less than 5) were discharged during an adhesion. This suggested that nematocysts of planulae do not discharge unless the corresponding cnidocils contact sabellid surfaces.

Planulae released both everted and undischarged nematocysts when flattened between slide and coverslip. The nematocysts were of a single type which did not conform clearly to any described for the family Proboscidactylidae (Russell, 1938; Hand, 1954; Calder, 1970). They appeared to be basatrichous haplonemes (Fig. 5). The nematocyst capsules left on severed pinnules and sabellid tube fragments appeared identical to those released from flattened planulae.

Behavior prior to attachment

Planulae that contacted severed *Schizobranchia* pinnules attached to the pinnules immediately. However, they often swam or crawled along paths that were almost, but not quite, tangential to severed pinnules without altering their direction or velocity as they passed the pinnules. Campbell (1968) observed that in the vicinity of an intact, extended sabellid, the currents passing through the crown of tentacular cirri greatly exceed the swimming velocity of *Proboscidactyla* planulae. My observations of planulae near severed pinnules and Campbell's result both suggest *Proboscidactyla* planulae take no active role in initiating contact with a suitable host.

Stimulus for attachment

To determine whether mechanical stimulation caused by contact with a ciliated surface might trigger nematocyst discharge and adhesion, a freshly excised portion of the branchial basket from a tunicate, *Chelyosoma productum,* and a piece of the lamellae from a scallop *Chlamys hastata*, were placed in each of 2 Syracuse dishes containing large numbers of planulae (about 250 in the first trial and 100 in the second). No planulae adhered to the ciliated ascidian or mollusc tissues. When severed *Schizobranchia* pinnules were added, planulae attached to the pinnules in large numbers. The presence of active cilia on a surface apparently does not, in itself, elicit nematocyst discharge.

Unattached planulae in crowded Syracuse dishes also did not adhere to fragments of *Schizobranchia* tube, although they frequently encountered the fragments. Such planulae failed to adhere even when squeezed lightly between 2 fragments of tube or a fragment of tube and the bottom of this dish. On the other hand, pieces of the collar folds, short lengths of contiguous body segments, and isolated portions of the ventral shields of *Schizobranchia* placed one at a time in Syracuse dishes containing planulae appeared to stimulate attachments as effectively as pinnules.

The great specificity of the attachment response suggest that a chemical stimulus found only on the body surfaces of sabellids is necessary to elicit nematocyst discharge in unattached *Proboscidactyla* planulae. I was unable to distinguish whether this chemical stimulus is an insoluble surface coating on the pinnules that the cnidoblasts detect only if they contact it directly, or a diffusable substance that triggers nematocyst discharge only in conjuction with a mechanical stimulus. Simple mechanical stimuli applied in the presence of mucus solutions or tissue "juices" from *Schizobranchia* failed to elicit a nematocyst response, but these solutions may have had toxic qualities that inhibited a nematocyst response.

Attachment to pinnules of a non-host sabellid species

Near Friday Harbor, *Schizobranchia insignis* is the most abundant sabellid species. *Eudistylia vancouveri* is morphologically similar to *Schizobranchia,* fairly common, and occurs in the same areas. However, *Eudistylia* tubes never bear *Proboscidactyla* colonies. Colonies can be successfully exchanged from one *Schizobranchia* specimen to another, but they regress when transplanted to *Eudistylia* (Strickland, 1971). Experiments comparing the tendencies of planulae to settle on *Schizobranchia* and *Eudistylia* pinnules revealed a clear preference for *Schizobranchia* although *Eudistylia* regularly stimulated attachment also (Fig. 6A and B). In A, the separate-dish design, 25 planulae and 10 *Schizobranchia* pinnules were placed in each of 4 culture dishes 2 cm in diameter. Four similar dishes were prepared with *Eudistylia,* rather than *Schizobranchia,* pinnules. In B, the competitive design, 25 planulae, 5 *Schizobranchia* pinnules, and 5 *Eudistylia* pinnules were placed together in each of 3 dishes. In both experiments the number of planulae that had attached to the *Schizobranchia* pinnules by 1, 2, 4, and 6 hr was significantly higher at the 99% confidence level (minimum value of t in Student test was 4.4 with 5 degrees of freedom). Nevertheless, in every trial or trial-pair some planulae attached to *Eudistylia* pinnules.

Elongation following attachment

Shortly after attaching to a pinnule, *Proboscidactyla* planulae lengthened along their longitudinal axes to a much greater extent than unattached planulae ever did

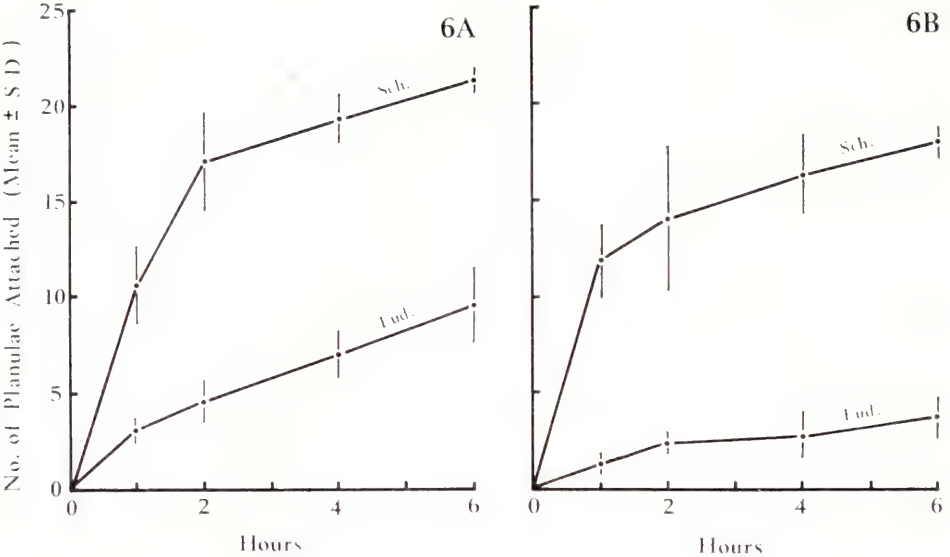
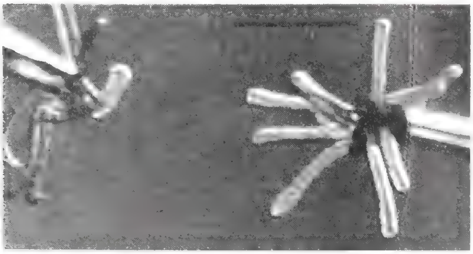


FIGURE 6. Experiments comparing planula settlement on pinnules from *Schizobranchia* and *Edistylia;* (A) separate dish design, (B) competitive design. Time elapsed following the addition of 25 planulae to each trial dish is plotted on the abscissas. The ordinates designate the mean number of planulae attached in all trials for each experimental design.

(Fig. 7). During elongation the cavity within the planula narrowed and sometimes nearly disappeared. Simultaneously the greatest diameter of the elongating planula shifted from anterior to posterior. The greater thickness of the posterior entoderm probably accounted for the posterior bulge of maximally elongated planulae (Fig. 7). The orientation of attachment (anterior, posterior, or lateral) had no visible effect on the shape of a planula upon elongation.

Data were obtained on the time between attachment and complete elongation for 25 planulae. Elongation required from 1.3 to 11 min ($\bar{x} = 4$ min, S.D. = 2.7).



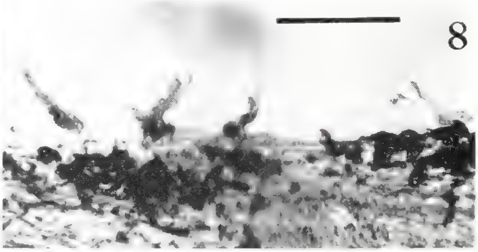


FIGURE 7. Elongated planulae after 12 hr adhesion to the fragment of tentacular cirrus held by the suction pipette on the right. The planula on the left had been attached for 1 min and had not yet elongated when the photograph was made; scale bar = 0.5 mm.

FIGURE 8. Newly metamorphosed *Proboscidactyla* polyps on the rim of a *Eudistylia* tube; scale bar = 0.5 mm.

The planulae that attached to severed *Eudistylia* pinnules during the inter-species comparison experiments (Fig. 6) elongated, but did so more slowly than planulae attached to *Schizobranchia* pinnules. Once elongated, attached planulae remained so in excess of 24 hr. By this time severed *Schizobranchia* pinnules had largely decomposed, although severed *Eudistylia* pinnules remained in good condition.

Planulae attached to the intact pinnules of a small *Schizobranchia* specimen (removed from its tube to prevent transfers to the tube rim) remained elongated in excess of 48 hr. These planulae and those that remained attached to pinnules instead of transferring to tubes never metamorphosed. Controls which completed their transfers almost always began metamorphosis within 12 hr. Elongation appeared to be associated exclusively with attachment to pinnules. Following transfer, planulae always rounded up within a few minutes. Transfer appears to initiate planula metamorphosis.

Desensitization to pinnules following attachment

Planulae swept into the crowns of intact sabellids only occasionally attached to more than one pinnule, although the curling and writhing of the worm's crown frequently brought the newly-attached planula into contact with several pinnules. Fifty-two planulae that had been attached to severed pinnules for 10–12 hr all failed to adhere when maneuvered into contact with freshly severed tentacular cirri from *Schizobranchia,* although 40 of them later transferred to tube fragments (Table 1). On 5 occasions individual planulae were touched with a second pinnule at 0.5 min intervals after attachment. Two planulae failed to adhere to the second pinnule on the first trial at 0.5 min. Two others adhered at 0.5 min and 1.0 min, but not at 1.5 min. None of these planulae had elongated appreciably before becoming desensitized to pinnule contacts. (About 1.3 min was the minimum time required to achieve full elongation in any of the 25 planulae timed). The fifth planula adhered to a proffered pinnule at 2.0 min, but not at 2.5 min. It required even longer, 9 min, to elongate and did not elongate completely. Thus desensitization occurs before elongation and the minimum time to desensitization is approximately 0.5 min.

TABLE I

Comparison of Schizobranchia *pinnules and tube surfaces as stimuli for the adhesion of attached planulae.*

Order of test surface application in each trial series	Number of planulae tested in each trial series (total 52)	Number of planulae adhering to each test surface (Total 40)				Number of planulae failing to adhere (total 12)
		pinnule	inside	mid-layer	outside	
pinnule, inside, outside	30	0	5		18	7
pinnule, outside, inside	9	0	0		7	2
pinnule, mid-layer, outside	3	0		0	2	1
pinnule, outside, mid-layer	10	0		1	7	2

Twenty-one planulae pulled off pinnules after 10–12 hr of attachment shortened up and resumed swimming after approximately 5 min. Within 10 min all of these planulae had recovered the ability to attach to *Schizobranchia* pinnules. Pinnule contact apparently must be maintained to prevent planulae from discharging nematocysts in response to new pinnule contacts.

Acquisition of a nematocyst response to worm tube

After becoming desensitized to pinnule contacts, attached *Proboscidactyla* planulae eventually begin to adhere when brought into contact with sabellid tube material. This change in effective stimulus for nematocyst discharge will be termed respecification. Under natural conditions attached planulae can only contact worm tube when the worm withdraws its crown of tentacular cirri. To estimate the time required for a newly attached planula to become responsive to worm tube I touched individual planulae to fragments of worm tube at intervals ranging from 1 min to 45 min after attachment. Out of a total of 20 transfers, the 2 earliest occurred 4 and 6 min after attachment.

An attempt to obtain a more accurate estimate of the time required for respecification was made by touching individual planulae to worm tube fragments at 2 min intervals, starting 2 min after attachment. However, the planulae subjected to these manipulations usually came off their pinnules or failed to adhere to the tube fragment within 30 min. Table II displays data from the 6 trials in which planulae adhered to tube fragments. Some of the first adhesions were not tenacious enough to result in a transfer, but eventually 5 out of the 6 planulae transferred. The one exception was injured accidentally before its 10 min trial. None of these planulae discharged nematocysts until they had been attached for at least 6 min. All 6 planulae had been elongated for at least 3 min before first adhering to a worm tube.

These and other observations suggested that elongation was associated with, and ordinarily preceded, respecification. However, 4 planulae that were substantially less than fully elongated nevertheless transferred successfully. Respecification and elongation may begin with the same stimulus or depend upon a common physiological event, but complete elongation is not a prerequisite for transfer.

Table II

Time requirement for respecification.

Elapsed time between attachment and elongation of each of 6 planulae (min)	Results of trials on tube at 2 min intervals (min after attachment)	
	Adhesion without transfer	Adhesion followed by transfer
2	6	8
2.5	8, 10, 12	18
11		14
3.5	8	
1.5		12
2.5		6

Orientation of attachment and transfer

In many, but not all, of the observed attachments, a point at or to one side of the anterior end of the planula adhered to the pinnule. However, by using a manipulator to maneuver the severed pinnules posterior attachments were readily obtained. Promptly after contact, planulae attached by the pointed posterior end often toppled over and adhered by one side, which suggests that physical instability may account for the comparative rarity of posterior attachments. Planulae that remained attached by the posterior and elongated normally, the enlarged end being the posterior as usual. On 6 occasions I noticed elongated planulae that had attached by their posterior ends after being swept into the plumes of intact sabellids.

Although many planulae, having attached by their anterior ends, adhered by their posterior ends during transfer, again this was not always the case. Often they adhered to worm tube fragments by one side. Four planulae, having attached to pinnules by their posterior ends, subsequently adhered to tube fragments by their anterior ends and transferred. The absence of a requirement for a particular orientation during either elongation or transfer suggests either that two populations of nematocysts are intermingled, or that nematocysts from all parts of the epidermis are functionally identical.

Stimulus for transfer

The data presented in Table I suggest that the outside of *Schizobranchia* tubes stimulates transfer far more effectively than does the inside or a middle layer. In obtaining these results the responsiveness of each attached planula to pinnule contacts was first tested by touching it to a short section of isolated *Schizobranchia* tentacular cirrus. Having obtained a negative response, the planula was then touched to either the inner surface of a *Schizobranchia* tube fragment or a middle layer of a "peeled" tube fragment. If the planula failed to transfer it was touched to the outer surface of another fragment of the same tube. With other planulae the same stimuli was applied in reverse order.

To determine whether attached and respecified *Proboscidactyla* planulae might adhere relatively non-selectively, 3 non-sabellid substrates from the mooring float community were tested: the tunic of *Cheliosoma productum*, test of the ectoproct *Membranipora tuberculata*, and an empty barnacle shell removed from the outside of a sabellid tube.

None of the 5 planulae tested on each of the 3 non-sabellid substrates adhered, although all 15 subsequently transferred when touched to a fragment of *Schizobranchia* tube. The stimulus for transfer, whether a film of microorganisms or not, appeared to be exclusively a property of sabellid tube material.

Individual planulae successfully transferred to fragments of *Eudistylia* tube on numerous occasions, sometimes after attachment to severed *Eudistylia* pinnules. The behavior of planulae that attached to the pinnules of small intact specimens of *Eudistylia* was indistinguishable from that of control planulae on *Schizobranchia;* they elongated, transferred, and metamorphosed into minute one-tentacled polyps (Fig. 8). Clearly *Eudistylia*, although not a host for *Proboscidactyla*, can induce

Proboscidactyla planulae to settle whereas other surfaces found in the same localities cannot.

Discussion

In comparative settling experiments, *Schizobranchia* pinnules were preferred to *Eudistylia* pinnules, but *Proboscidactyla* planulae attached to pinnules of both sabellid species. Once attached, the responses of planulae to *Eudistylia* were qualitatively indistinguishable from the responses elicited by *Schizobranchia.* None of the other test substrates induced planulae to adhere even momentarily. Planulae metamorphosed on intact specimens of *Eudistylia* in the laboratory and there is no reason to suspect that they fail to do so in the field. Apparently the deficiencies of *Eudistylia* as a host for *Proboscidactyla* are not manifested until after metamorphosis.

Nematocyst responses play a central role in the settlement of *Proboscidactyla* planulae and constitute a major part of the behavioral repertoire of these larvae. Free-swimming planulae ordinarily discharge nematocysts only upon contact with the pinnules of a sabellid. Contact with non-sabellid, ciliated surfaces does not elicit nematocyst discharge, although the mechanical stimulus is probably comparable to that provided by sabellid pinnules. However, contact with any body surface of *Schizobranchia* results in attachment. *Eudistylia,* although closely related to *Schizobranchia* and morphologically very similar, is less effective in stimulating planulae to attach. Following attachment and respecification, planulae again respond specifically, this time discharging nematocysts upon contact with the outer surfaces of sabellid tubes. These results suggest that specific chemical stimuli are needed to elicit nematocyst discharge in *Proboscidactyla* planulae.

Planulae discharge nematocysts individually at sites of contact rather than en masse. This indicates either that direct contact with a chemically stimulating surface is required to excite a cnidoblast, or that mechanical as well as chemical stimulation is needed. The first possibility has never been described, but the second, dual stimulation, has been demonstrated in some cnidarians by applying chemical and mechanical stimuli separately and in combination (Pantin, 1942; Jones, 1947; Burnett, Lentz, and Warren, 1960). I was unable to excite the nematocysts of *Proboscidactyla* planulae with simple mechanical stimuli in the presence of mucus solutions or tissue "juices" from *Schizobranchia.* Therefore it is conceivable that contact chemoreception alone mediates the nematocyst response in this case, although these negative results do not constitute a conclusive demonstration.

After about 30 sec of attachment to a pinnule, a *Proboscidactyla* planula becomes desensitized to additional pinnule contacts. Subsequently, the nematocyst response is respecified, sabellid tube replacing pinnule as the effective stimulus. Intercellular controls of nematocyst function must be postulated to explain desensitization and respecification. There may be two functional classes of nematocysts, one responsive to pinnule and the other to worm tube. Because any part of a planula can adhere to either pinnule or worm tube, it follows that if two types of nematocysts exist, they must both be distributed uniformly over the epidermal surface. Prior to attachment, the pinnule nematocysts would be ready to discharge while the tube nematocysts would be inhibited. After attachment, owing to changes in physiological state throughout the cnidoblast population, the tube nematocysts would become readied and the pinnule nematocysts inhibited. Alternatively, there may be only one type of nematocyst in *Proboscidactyla* planulae. Physiological events associated with attachments to a pinnule would respecify the remaining cnidoblasts to discharge only to worm tube.

In the nematocyt literature the term "independent effector" has been used in different ways. Early authors used the term specifically to indicate that nematocysts are not directly excited by the nervous system (Parker and Van Alstyne, 1932; Pantin, 1942; Jones, 1947). More recently authors have described examples of endogenous nematocyst controls operating through unidentified physiological pathways as evidence against the independent effector hypothesis (Davenport, Ross, and Sutton, 1961; Ross and Sutton, 1964; Sandberg, Kanciruk, and Mariscal, 1971; Oshida, 1972). It is possible that the nematocysts of *Proboscidactyla* planulae are independent effectors in the original restricted sense, although their function is clearly subject to endogenous controls.

Although innervation of some cnidoblasts has been histologically demonstrated (Westfall, 1970; Westfall, 1971), the settlement behavior of *Proboscidactyla* planulae can be explained without assuming that such innervation exists. Desensitization probably requires a minimum of about 30 seconds to complete and sometimes takes over 2 minutes. Respecification requires several minutes. Nervous (or epithelial) conduction would transmit the message of attachment from one end of the 250 μ planula to the other in a few milliseconds. If nervous conduction is involved, there presumably are slow steps at the receptor sites, cnidoblasts, or both to account for the observed delays. On the other hand, slow conveyance of information by a non-nervous process might largely account for the considerable time lag before desensitization and, in combination with a slow step in the cnidoblasts, for the still greater delay prior to respecification. Both desensitization and respecification are reversible changes maintained only by continuous contact with a sabellid pinnule. This suggests that information is continuously transferred from the attachment site. It is possible that a metabolite diffuses from the attachment site through the tissues of the planula and triggers the sequence of events that follow attachment.

Planulae that attached and did not transfer remained elongated for extended periods of time, but never metamorphosed. After transferring to the rims of sabellid tubes, planulae that had previously been attached to pinnules promptly rounded up and subsequently commenced metamorphosis. These observations indicate that the stimulus for metamorphosis is not the same as the stimulus for attachment, desensitization, elongation, or respecification. The stimulus that initiates metamorphosis and the stimulus for adhesion and transfer to sabellid tube could be one and the same. Elongation, while not a prerequisite for transfer, may be a behavioral adaptation that increases the probability of transfer by increasing the likelihood of contact with the sabellid tube rim.

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SUMMARY

Planulae of the symbiotic hydroid *Proboscidactyla flavicirrata* settle on the rims of sabellid tubes by adhering sequentially and specifically to two different substrates. For both steps, nematocysts are the agents of adhesion. Although the cnidoblasts of these planulae require local excitation, they are subject to endogenous controls.

Upon being swept into the plumes of tentacular cirri that sabellids extend from their tubes, planulae attach to individual pinnules (branches of the tentacular cirri) by discharging nematocysts. Within 2 min following attachment, the planulae cease discharging nematocysts in response to additional pinnule contacts. Planulae transfer to the rims of sabellid tubes by adhering with nematocysts when the sabellids retract their plumes. Cnidoblasts of planulae only become responsive to contact with sabellid tube after 4 min or more of continuous attachment to pinnules. Transfer initiates metamorphosis.

Planulae are capable of settling and metamorphosing on *Eudistylia vancouveri*, a sabellid species that is sympatric with the normal hosts of *Proboscidactyla*, but will not support colonies. Apparently the deficiencies of *Eudistylia* as a host are manifested only after planula metamorphosis.

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NOTE ADDED IN PROOF

The identification of the planula nematocysts given in this report is in error. They are microbasic euryteles not basitrichs.

Reference: *Biol. Bull.*, **147**: 586–593. (December, 1974)

CYTOPLASMIC DNA IN SEA URCHIN OOGENESIS STUDIED BY ^{3}H-ACTINOMYCIN D BINDING AND RADIOAUTOGRAPHY

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This study is concerned with the time at which cytoplasmic DNA of the sea urchin oocyte replicates in relation to the stage of oocyte development. Oocytes at many different states of development, as well as mature eggs, are found in mature sea urchin ovaries. They range in size from the smallest primary oocyte to the largest terminal oocytes. Since all different stages of oocyte development are present in the ovary, the events of oogenesis may be studied in a sequential manner.

The method chosen to detect the presence of cytoplasmic DNA in relation to the stage of oocyte development was ^{3}H-actinomycin D incubation of sectioned material, followed by radioautography. It is well established that actinomycin D binds specifically to DNA (Muller and Crothers, 1968) and that this binding occurs on tissue sections (Ebstein, 1967, 1969). Once the ^{3}H-actinomycin D binds specifically to DNA on the section, the location of the ^{3}H-actinomycin D–DNA complex can be detected by radioautography. We thus have a method for localizing all nuclear and cytoplasmic DNA in tissue sections in relation to cell structures. This method allows us to compare the amount of cytoplasmic DNA detected by ^{3}H-actinomycin D binding at progressive stages of oocyte development.

MATERIALS AND METHODS

Living supplies of the purple sea urchin, *Strongylocentrotus purpuratus,* were obtained from Pacific Biomarine Company, Venice, California. Ovaries of mature females were fixed in Carnoy's solution (ethanol : acetic acid, 3 : 1) for two hours. They were passed through graded alcohols, cleared in toluene and embedded in paraffin. Sections were cut at 5 microns, deparaffinized in xylol and hydrated through a graded alcohol series.

Control enzyme and TCA extraction of DNA and RNA

Controls were necessary to check the specificity of the ^{3}H-actinomycin D binding under the experimental conditions used. Controls consisted of slides from which all DNA had been extracted prior to incubation with ^{3}H-actinomycin D, either by treatment of the slides with DNase, using the method of Deitch (1966) or by extraction with hot trichloroacetic acid (TCA), using the method of Pearse (1961). As an additional control, RNase extractions, using the method of Amano (1962) were performed on another series of slides. The specificity of the above extraction procedures was confirmed by Azure B staining for DNA and RNA using the cytochemical staining method of Flax and Himes (1952).

Incubation with ^3H-actinomycin D

To determine the location of DNA in the oocytes, slides were incubated in ^{3}H-actinomycin D. The method of Ebstein (1967, 1969) as modified by Aczel and Enesco (1973) was used. This method consists of incubating the slides with ^{3}H-actinomycin D (5 μCi/ml) for two hours in a moist chamber in the dark, then rinsing the slides in distilled water and in a graded alcohol series to 70% ethanol to remove any ^{3}H-actinomycin D which is not specifically bound to DNA. The ^{3}H-actinomycin D (specific activity 3.7 Ci/mM) was obtained from Schwartz-Mann Company, Orangeburg, New York.

Radioautography and staining

Slides were air dried following ^{3}H-actinomycin D incubation to prepare them for radioautography. The slides were then dipped in Kodak NTB2 liquid nuclear track emulsion, using the method of Kopriwa and Leblond (1962). After sufficient exposure (10 days) they were developed in D170 developer (Young and Kopriwa, 1964). The slides were then stained following radioautographic development with either cresyl violet or toluidine blue. The staining procedures followed were those selected by Thurston and Jofts (1963) for their compatibility with the radioautographic emulsion. After staining, the slides were dehydrated and mounted in Permount.

Staging of oocytes

The dimensions of the oocyte nucleus and cytoplasm as sectioned through their largest diameter were measured with the aid of a calibrated ocular micrometer. On the basis of these measurements, oocytes were classified into ten numerical stages, as described in the results section. Numerical stages 1 and 2 correspond to primary oocytes, stages 3 to 6 are growing oocytes and stages 7 to 10 are terminal oocytes using the classification defined by Cowden (1962) and by Esper (1965). At least 10 measurements were made to define each stage.

Histometrics and grain counts

Oocytes sectioned through their largest diameter were selected for this study. The nuclear and cytoplasmic diameter of these oocytes was measured, and the nuclear and cytoplasmic area was calculated. Using the measured values of nuclear and cytoplasmic diameter, nuclear volume and total cell volume were calculated. Cytoplasmic volume was then obtained by subtracting nuclear volume from total cell volume. The number of grains per $1000\mu^2$ cytoplasmic area was determined on the same sections used for histometrics. The grain counts, originally expressed in relation to area, were then transformed to express the total number of grains expected in relation to total cytoplasmic volume.

TABLE I

Grain counts per 1000 μ^2 over nucleus and cytoplasm at 3 stages of oocyte development, corrected by subtraction of background fog. The amount of radioactivity in non-extracted, RNase, DNase and TCA extracted tissue is presented. The data for stages 4 to 10 is essentially the same.

	Stage	No extraction	RNase extraction	DNase extraction	TCA extraction
Nucleus	1	266	282	8	2.9
	2	200	203	5.9	5.4
	3	130	153	0	0.5
Cytoplasm	1	66	77	4.5	1.7
	2	60	78	3.2	5.7
	3	43	48	2.8	1.4

RESULTS

It was first necessary to establish that ^{3}H-actinomycin D was binding specifically to DNA under our experimental conditions. As would be expected, radioautographic examination showed that ^{3}H-actinomycin D labelling was heavy over nuclei at all stages of oocyte development. Label appeared over the cytoplasm as well. Evidence of specific ^{3}H-actinomycin D binding to DNA in both nucleus and cytoplasm of the sea urchin oocytes is presented in Table I. This table presents the number of silver grains per 1000 μ^2 over nuclei and over cytoplasm in the radioautographs, which in turn represents the amount of ^{3}H-actinomycin D binding to DNA. As control data, grain counts from slides extracted with RNase, DNase, and hot TCA are presented. The grain count data for the RNase extracted slides is not significantly different from the non-extracted slides. Thus, RNase extraction does not influence ^{3}H-actinomycin D binding. In contrast, on slides where all DNA has been removed by extraction with DNase or with hot TCA, the grain counts were reduced to very low levels, not significantly above background. This data clearly indicates that there is no ^{3}H-actinomycin D binding in the absence of DNA. The effectiveness of the RNase, DNase and TCA extractions was further confirmed by the absence of selective staining when slides treated by these extraction methods were stained with Azure B bromide, which stains DNA and RNA selectively. The pattern described above shows that no ^{3}H-actinomycin D binding takes place when all DNA has been removed from the sections. Table I presents representative grain counts from stages 1 to 3. The data for stages 4 to 9 follow exactly the same pattern.

Table II presents the direct measurements of nuclear and cytoplasmic area used to define the 10 stages of oocyte growth. From this data, the total cytoplasmic volume at each stage of oocyte growth was calculated. The data on Table II shows that there is a 26 fold increase in cytoplasmic volume between stages 1 and 10.

We now turn to the ^{3}H-actinomycin D binding data for the cytoplasm, the primary concern of this study. Table II presents the grain counts, representing ^{3}H-actinomycin D binding to cytoplasmic DNA, in a standard unit measure of grains per 1000 μ^2 cytoplasmic area. This value decreases markedly from stage 1 to 5, suggesting a dispersal of cytoplasmic DNA correlated with the marked increase in size of the oocyte between these two stages. In contrast, from stage 6 to stage 10 the grain counts per 1000 μ^2 remains almost constant, despite the continued growth of the oocyte.

Since the oocyte is a three dimensional sphere, we will now use the above data to obtain an estimate of the amount of ^{3}H-actinomycin D binding to cytoplasmic DNA in the total cytoplasmic volume. Table II shows the total cytoplasmic volume at each stage of oocyte growth. Table II also shows the amount of ^{3}H-actinomycin D binding calculated to occur in relation to total cytoplasmic volume. This latter figure provides a comparative estimate of the relative amounts of cytoplasmic DNA present at various stages. The data shows that there is a 4-fold increase in ^{3}H-actinomycin D binding to total cytoplasmic DNA during oocyte development.

At each stage of oocyte development we have now determined two variables: cytoplasmic volume, and total grain counts calculated in total cytoplasmic volume Figure 1 expresses each of these variables in relation to stage of oocyte development. The two ordinates of Figure 1 are drawn to separate and arbitrary scales, for the purpose of comparing the shape of the two curves. This manner of presentation emphasizes the points of similarity between the two curves. Figure 1 shows that the most rapid increase in the amount of cytoplasmic DNA (grain counts) occurs in the primary and young growing oocyte (stages 1 to 5). There is a stabilization in the amount of cytoplasmic DNA at stage 5, but no marked increase between stages 5 to 10. When the volume increase of the oocyte is plotted on the same graph for comparison, we see that the increase in cytoplasmic volume starts slowly from stages 1 to 3, then rises sharply from stages 3 to 5, plateaus briefly at the stages 6 and 7, and then increases steadily from stages 7 to 10.

During the first stages of oocyte growth, the plots for increase in cytoplasmic volume and for increase in cytoplasmic DNA appear quite similar. However, after

TABLE II

The numerical stages of oocyte growth are defined in relation to the size of the nucleus and cytoplasm. Grain count data is expressed in relation to unit cytoplasmic area, total cytoplasmic area and total cytoplasmic volume.

Stage	Nuclear area μ^2	Cytoplasmic area μ^2	Grain count per 1000 μ^2 cytoplasmic area	Cytoplasmic volume μ^3	Number of grains calculated per total cytoplasmic volume
1	110	900	266	24,300	362
2	135	1080	200	32,000	451
3	500	1250	130	49,100	652
4	850	5000	49	326,000	1096
5	900	6000	35	421,200	1208
6	1000	6500	29	483,800	1221
7	1100	6500	27	483,700	1253
8	1150	7200	25	573,200	1306
9	1200	9000	25	743,700	1289
10	1250	9500	25	867,900	1325

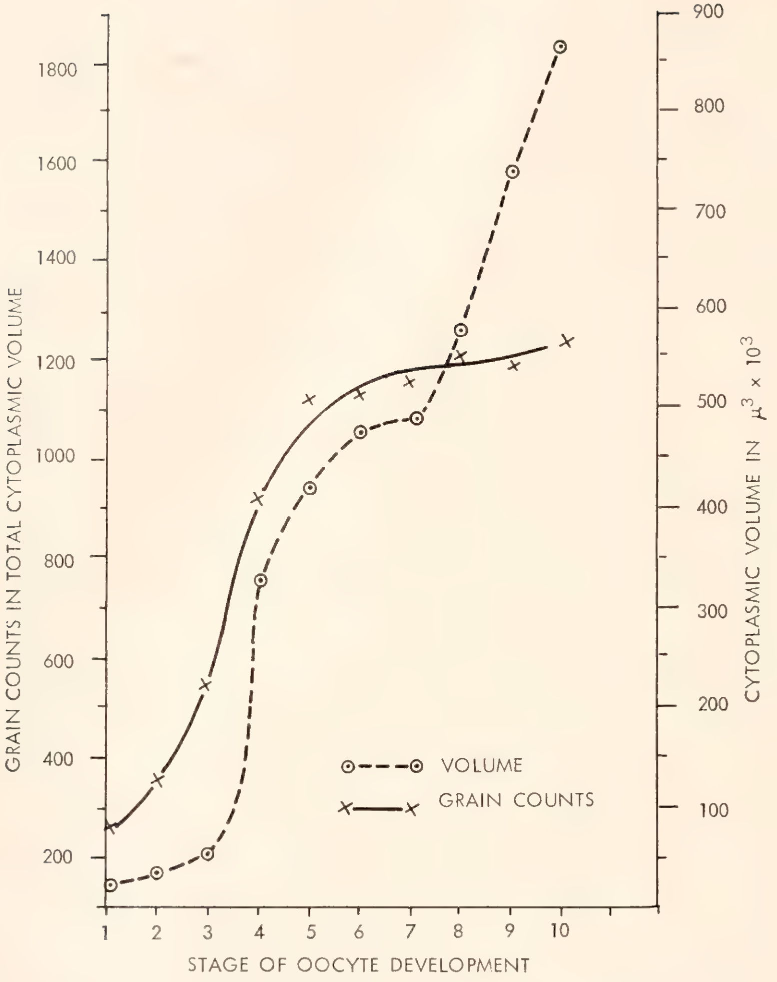

FIGURE 1. Grain counts on radioautographs, which represent the amount of ^{3}H-actinomycin D binding to cytoplasmic DNA at successive stages of sea urchin oocyte development are plotted on the left ordinate. Increase in cytoplasmic volume is plotted on the right ordinate. Arbitrary scales were chosen for the purpose of comparing the shape of the two curves. This comparison shows that increase in mitochondrial DNA parallels increase in cytoplasmic volume only in the early and growing oocyte stages.

stage 7 all similarity disappears. The oocyte continues to increase in volume, but there is no further increase in cytoplasmic DNA.

DISCUSSION

No controlled *in vitro* studies of ^{3}H-actinomycin D binding to sea urchin oocytes had been carried out prior to the present study. *In vivo* incubation studies by Greenhouse, Hynes and Gross (1971) have shown that actinomycin D inhibits RNA synthesis in developing sea urchin embryos, and have established that ^{3}H-actinomycin D readily penetrates into living embryos. Radioautographic localization of the ^{3}H-actinomycin D binding sites showed that silver grains were distributed over the cytoplasm as well as over the nuclei of the early cleavage embryos. The authors interpret the cytoplasmic label as cytoplasmic DNA. Their experimental design did not permit confirmation with DNase extracted controls.

The amount of ^{3}H-actinomycin D binding which takes place in any experimental system is a function of both of the concentration of the ^{3}H-actinomycin D used and of the incubation time used (Simard, 1967). While ^{3}H-actinomycin D binding does not quantitatively reflect the absolute amount of cytoplasmic DNA present in the oocytes, we can use the actinomycin D binding data as a comparative estimate of the relative amount of cytoplasmic DNA available for the ^{3}H-actinomycin D binding at the various stages of oocyte development. The lack of ^{3}H-actinomycin D binding after DNase extraction in this study excludes any label being non-specifically bound to either nucleus or cytoplasm. Binding of ^{3}H-actinomycin D to cytoplasmic DNA has been reported in only one previous study (Aczel and Enesco, 1973), although several investigators have reported on ^{3}H-actinomycin D binding to nucleolar DNA (Simard, 1967, Camargo and Plaut, 1967).

It was first reported by Hoff-Jørgenson (1954) that sea urchin eggs contained large amounts of cytoplasmic DNA. This cytoplasmic DNA was first shown to be mitochondrial DNA by Piko, Tyler and Vinograd (1967), who also reported that one third of the cytoplasmic DNA in the sea urchin egg was associated with the yolk spherule fraction. These findings were subsequently revised by Berger (1968) who showed that DNA becomes associated with the yolk fraction in sea urchin eggs only as a result of contamination by the mitochondrial fraction. The results of Berger (1968), thus show that the "cytoplasmic" DNA of the sea urchin egg is entirely mitochondrial DNA. Dawid (1972) also cites evidence that the cytoplasmic DNA reported to occur in eggs of various species is entirely mitochondrial DNA. This is true not only for sea urchin eggs, but for frog eggs (Dawid, 1966) and for eggs of *Urechis caupo* as well (Dawid and Brown, 1970).

Mitochondrial DNA from mature sea urchin eggs has been isolated and characterized by Piko and associates (Piko *et al*, 1967, Pilo, Blain, Tyler and Vinograd, 1968). The replication of mitochondrial DNA during sea urchin oogenesis follows the same molecular replication pattern generally reported for mitochondrial DNA (Matsumoto, Kasamatsu and Piko, 1973).

In the present study, the fixation and staining methods necessary for radioautography are not suitable for morphological observation of mitochondria. Even though we cannot correlate silver grains directly with mitochondria, it is clear from the work of Berger (1968) that the mitochondria are the only DNA containing organelles in the cytoplasm.

The results presented here show that increases in the amount of cytoplasmic DNA, as detected by ^{3}H-actinomycin D binding, are confined to the preliminary and growing oocyte stages. No increase in cytoplasmic DNA takes place in the terminal oocyte stages. If we now interpret cytoplasmic DNA as mitochondrial DNA, how do these results correlate with our knowledge of mitochondria in oocytes?

We do know that the number of mitochondria in the sea urchin oocyte inceases markedly during oogenesis. Verhey and Moyer, (1967) report that there is an average of 393 mitochondria in a small oogonium, as compared to an average of 19,827 mitochondria in a mature oocyte. These figures are based on calculations from electron microscope data, and represent a 50 fold increase in the number of mitochondria from the earliest to the latest stage of oocyte development. This data does not tell us the time at which mitochondrial replication takes place, and provides no information about mitochondrial DNA.

These combined results of this and the other studies cited show that the synthetic activities of the sea urchin oocyte are sequentially arranged into specific stages of oocyte differentiation.

The primary and growing oocytes are characterized by intensive RNA synthesis and production of ribosomes (Verhey and Moyer, 1967) as well as by the increase in cytoplasmic DNA reported in this study. Intensive yolk synthesis takes place at later stages (Esper, 1962, 1965).

Dawid (1972) suggests that large numbers of mitochondria may accumulate in the egg as a storage product to support embryonic development during the rapid cleavage stages. Gustafson (1965) reports that the number of mitochondria remain constant in the sea urchin from the mature oocyte stage through gastrulation. It is clearly the oocyte mitochondria which support the early development and differentiation of the sea urchin embryo. The oocyte mitochondria, containing mitochondrial DNA are thus stored in the oocyte for distribution among the cells of the growing embryo. These results show that mitochondrial replication takes place primarily in the early oocyte stages.

SUMMARY

The relative amount of cytoplasmic DNA in sea urchin oocytes was studied at ten successive stages of oocyte differentiation in the sea urchin, *Strongylocentrotus purpuratus*. The cytoplasmic DNA was selectively localized by means of ^{3}H-actinomycin D binding on 5 μ sections of the ovaries. The specificity of the ^{3}H-actinomycin D binding to DNA was established using DNase extracted control slides. The results show that cytoplasmic DNA increases continuously during the primary and growing oocyte stages. Although oocytes at later stages of differentiation continue to increase in volume, there is no increase in cytoplasmic DNA during these later stages. The cytoplasmic DNA observed in this study is interpreted as mitochondrial DNA.

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FACTORS AFFECTING MUSCLE ACTIVATION IN THE HYDROID *TUBULARIA*

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The control of muscle contraction in coelenterates is complex and not well understood. For example, some muscles of sea anemones are activated by a diffuse, all-or-nothing conducting system, probably the nerve net of the column and mesenteries (Pantin, 1935a, 1935b, 1952; Pickens, 1969; Robson and Josephson, 1969). There are requirements for facilitation such that a single impulse in the conducting system initiates little or no mechanical response while a pair of impulses at a suitable interval evokes a moderately rapid twitch. During repetitive stimulation the mechanical response both facilitates and sums. The site of facilitation appears to be between the conducting system and the muscle membrane, probably at the neuromuscular synapse (Pantin, 1935a; Josephson, 1966; Robson and Josephson, 1969). While these features seem conventional, other properties of the muscles are not. The same muscles in anemones which give rapid contractions can also contract very slowly. The slow contractions frequently occur spontaneously and can be slowly propagated along the muscle sheet (Batham and Pantin, 1954; Ewer, 1960; Pantin, 1965a). Conducting systems have been identified which initiate and inhibit slow contractions (McFarlane, 1974) but in general the mechanisms coordinating slow contractions are unclear.

In scyphomedusae, bell contractions are initiated by impulses in an all-or-nothing conducting system, here almost certainly a nerve net (Horridge, 1954). Facilitation is less pronounced in medusae and a long refractory period of the muscle prevents appreciable summation (Bullock, 1943; Horridge, 1955). Twitches of the bell musculature can be augmented by contemporary activity in a second conducting system (Horridge, 1956), but the mechanisms of this augmentation are unknown.

Muscle contraction in hydrozoans has been less studied than in scyphozoans or anthozoans, largely because the small size of most hydrozoans is not conducive to tension measurement. In hydra, where tension recordings are available, longitudinal contraction of the column is peculiarly biphasic (Josephson, 1967). Excitation in a column conducting system initiates a rapid tension increase followed by a slower tension rise. Whether both tension components are generated by the same contractile cells or how either component is initiated is unknown.

Thus coelenterate muscle contraction varies with the pattern of impulses arriving at the muscle; the contraction may be influenced by activity in more than one conducting system; and muscle sheets, possibly even individual muscle cells, may produce both fast and slow contractions (*cf.* Horridge, 1956; Pantin, 1952, 1965b). Details about the mechanisms of muscle activation are obscure. Some of the uncertainties about the control of muscle contraction could be resolved with intracellular recording but the small size and contractility of coelenterate epitheliomuscular cells make intracellular recording a formidable task. One is left then with less direct measures, developed tension or extracellularly-recorded muscle action potentials, to use in analyzing the mechanisms controlling muscle contraction.

Electrical events which are probably muscle action potentials can be easily recorded from the hydroid *Tubularia* with surface suction electrodes. These potentials can be used to quantify muscle activation without having to directly record muscle tension. This paper considers some of the factors modifying the amplitude of these potentials and hence, by inference, the intensity of muscle activation.

Some background information on the morphological and functional organization of *Tubularia* would be appropriate at this point. *Tubularia* is a moderately large hydrozoan polyp with two sets of tentacles, a proximal set at the base of the hydranth and a distal set which encircles the mouth. The hydranth is borne on a long stalk. In mature polyps gonophores hang from stalks which arise just distal to the proximal tentacles. The spontaneous behavior of *Tubularia* results from the presence of a number of interacting pacemaker systems, the two most important of which are the neck pulse (NP) system in the distal stalk and the hydranth pulse (HP) system in the hydranth (Josephson and Mackie, 1965). The NP system produces electrical pulses (neck pulses = NP's) which typically occur as a sequence of single pulses interrupted periodically by a burst of pulses. Potentials from the HP system (hydranth pulses = HP's) occur as single pulses and pulse bursts. When the HP system fires it initiates coordinated closure of the proximal tentacles and weaker, synchronized closure of the distal tentacles.

Three conducting systems have been identified in *Tubularia* (Josephson, 1965) of which one, the distal opener system (DOS), is of interest here. The DOS courses through the stalk and hydranth. Activating the DOS initiates contraction of the aboral longitudinal musculature of the distal tentacles resulting in outward flaring (opening) of the distal tentacles. Activity in the DOS also inhibits the HP system and other pacemaker systems in the hydranth (Josephson and Uhrich, 1969; Josephson and Rushforth, 1973). Electrical potentials from the DOS can be recorded with surface suction electrodes or extracellular microelectrodes. In the stalk and most of the hydranth, DOS pulses (DOSP's) are small (50–200 μV), short (9–15 msec), all-or-nothing electrical events. In the vicinity of muscles activated by the DOS a second component appears in the DOSP. With an electrode on a distal tentacle or at the base of the distal tentacles the short, all-or-nothing pulse is followed by a slow, graded potential. This second DOSP component is thought to be a muscle action potential because its amplitude varies monotonically with that of observed tentacle flaring. Both the slow potential and tentacle opening defacilitate to the same degree during repetitive stimulation and both decline in parallel during anesthetization with excess magnesium (Josephson, 1965). The initial DOSP component is unchanged during repetitive stimulation (Fig. 1A) and its amplitude remains constant during anesthetization until it abruptly ceases to appear. Thus the initial DOSP component appears to be generated by the conducting system, the second component by the responding musculature. The amplitude of the second DOSP component is here used as a measure of the intensity of muscle activation.

MATERIALS AND METHODS

Tubularia were collected from the Woods Hole area. Animals were obtained from different places at different times of the year and, judging by the information provided by Miller (1969), included three species; *T. crocea* from the jetty at New Bedford, *T. spectabilis* from Woods Hole and the Cape Cod Canal, and *T. larynx* from the Cape Cod Canal. For the physiological parameters considered in this study, animals thought to be *T. larynx* and *T. spectabilis* were indistinguishable. *T. crocea* differs from the other two in having a longer, more active proboscis, in producing generally larger DOSP's and in other particulars described below.

The stimulating and recording methods are described in detail elsewhere (Josephson and Uhrich, 1969; Josephson and Rushforth, 1973). Briefly, animals were stapled to the bottom of a dish of sea water cooled to 16–18° C. A suction electrode on a gonophore or gonophore stalk was used to activate the DOS. The stimuli were 1 msec voltage pulses. Another suction electrode on the base of a distal tentacle recorded DOSP's and HP's. Recorded activity was displayed on a pen-writer or oscilloscope.

RESULTS

Defacilitation during repetitive DOS activation

The most obvious factor affecting DOSP amplitude is preceding DOS activity; the slow component of DOSP's shows marked defacilitation (Fig. 1A; Josephson, 1965). The time course of defacilitation was examined by stimulating the DOS at a set of interstimulus intervals ranging from 2 to 300 seconds. In each trial the intervals of the set were presented in random order. Results from four animals, each subjected to 20 sets of interstimulus intervals, are shown in Figure 2. In one of these animals the DOSP's reached maximum size with interstimulus

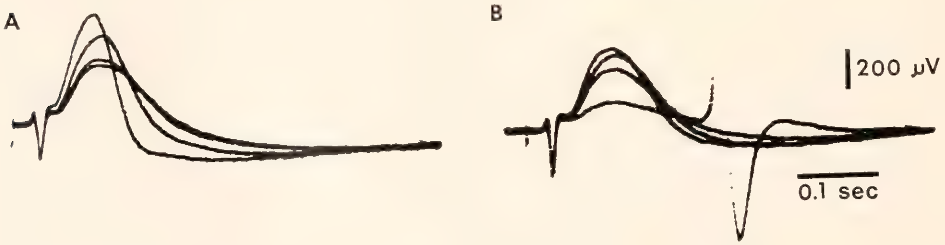

FIGURE 1. Depression of DOSP's during repetitive stimulation and following HP activity; (A.) defacilitation of the second component during stimulation at five-second intervals. The largest response is to the first stimulus which came after a long rest; subsequent responses became progressively smaller. (B.) DOSP's during and after an HP burst; the DOS was being regularly stimulated at 10-second intervals. The first DOSP, the one with the greatly reduced second component, occurred in the burst; the next three with progressively increasing amplitude came 7, 17, and 27 seconds after the burst. The first DOSP was followed by an HP (the potential halfway through the trace) which is poorly resolved at the high amplification used. Positive potentials are upward deflections. Note the constant amplitude of the first component.

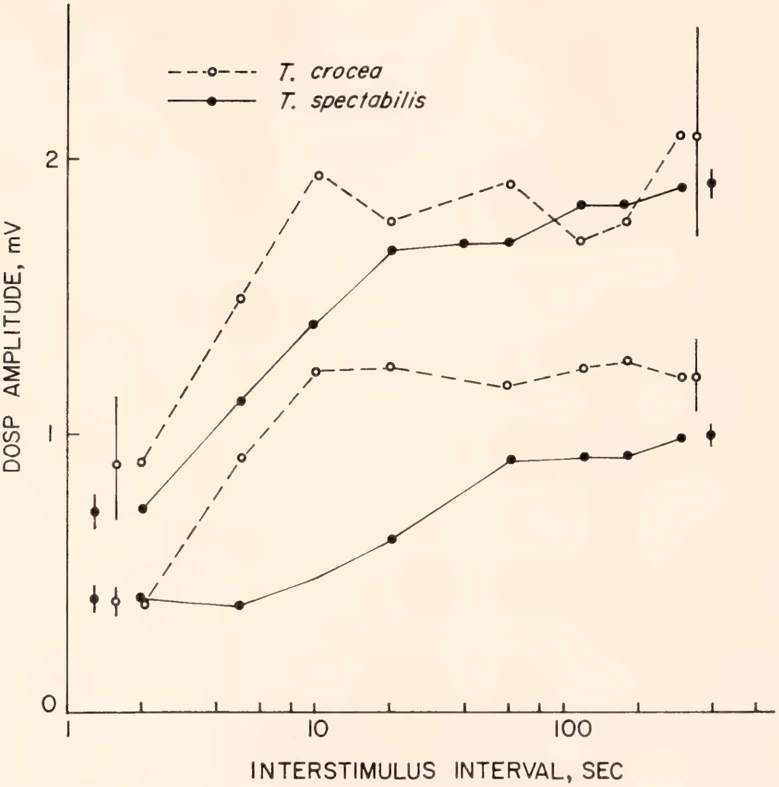

FIGURE 2. Amplitude of the DOSP second component as a function of interstimulus interval. Each point is the average of 20 determinations; standard errors for the first and last points are shown to the left and right of the curves. See text for further information.

intervals of ten seconds or more; in the other three, DOSP's continued to increase with increasing interval to the longest used. In the four animals the DOSP amplitude after a 300-second interval was 2.3 to 3.1 times greater than that with an interstimulus interval of 2 seconds.

The pattern of DOSP amplitude change during bursts of stimuli varies with the stimulus frequency. With interstimulus intervals of about five seconds or more, DOSP's decline monotonically during the burst until they reach an approximate steady state after three or four stimuli. At shorter intervals the DOSP depression sometimes overshoots and the response to the second stimulus may be the smallest of the series, subsequent DOSP's growing slightly until a steady state is reached (Fig. 3).

It might be noted that defacilitating effector responses are unusual in coelenterates; facilitating responses being more typical (*e.g.*, Pantin, 1935a; Bullock, 1943; Morin and Cooke, 1971). Another example of defacilitating muscle contraction, again from a hydroid, occurs in the lashing of dactylozooids from *Hydractinia* (Stokes, 1974).

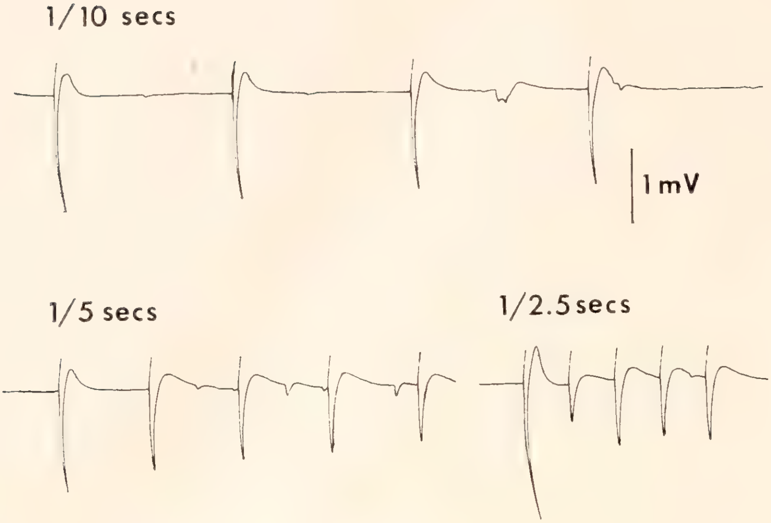

FIGURE 3. Defacilitation of the DOSP during repetitive stimulation, *T. crocea.* The short, upward deflections preceding the DOSP's are stimulus artifacts. In this and the other pen-writer records (Figs. 4, 6) positive potentials are downward deflections.

Depression following pacemaker activity

DOSP's closely following HP's tend to be smaller than those coming long after HP's (Fig. 4). As with defacilitation it is only the second component of the DOSP which is depressed (Fig. 1B). The time course of this depression was examined by stimulating the DOS at a regular frequency so that defacilitation reached a steady state and then determining the DOSP amplitude as a function of the interval between the DOSP and preceding HP activity.

Rather large sample sizes and therefore long recording sessions were required for reasonable temporal resolution. For example, the data of Figure 5A represents over 4 hours of continuous stimulation and recording. During long sessions the amplitude of recorded DOSP's often declines. This may be due in part to fatigue but probably more largely to slippage of the recording electrode or tissue damage beneath the electrode. To compensate for this drift the amplitude of a DOSP following HP firing was expressed as a percent of the DOSP size immediately before the HP activity. The stimulus frequency was one per 10 seconds and the stimuli were initially about twice DOS threshold. During long stimulation periods the DOS occasionally failed to fire following a stimulus. Each time there was such a miss the stimulus intensity was increased. If this eventually did not give faithful following, the stimulating electrode was repositioned and the run continued. Because of partial reduction in defacilitation, the DOSP was larger than normal following the first and sometimes the second successful stimulus after an unsuccessful one. For this reason only those instances were considered in which the control DOSP, that immediately before the HP activity, was preceded by two consecutive successful stimuli. Similarly if there was a miss following the HP activity, subsequent DOSP's in the post-HP interval were not included in the tabulated results. Another restriction was that the control DOSP had to be separated from the HP preceding it by more than 20 seconds, the major part of the HP effect on DOSP's being over by this time.

Results from the animals giving the two longest series are shown in Figure 5. It is apparent that DOSP depression by HP's is greater following long HP bursts (4 or more pulses) than after short bursts (2 or 3 pulses) and slightly greater after short bursts than following single HP's. Recovery from depression by HP's is at first rapid but there is some indication that recovery can continue for as long as 40 to 60 seconds. At long post-HP intervals the DOSP's are, on the average, larger than controls. This is probably because the DOSP's in these bins occur at post-HP intervals which are longer than is true for the average control DOSP.

HP depression of DOSP's is much more erratic in *T. crocea* than in the other species. In only a few of the more than 20 specimens of *T. crocea* examined was there clear depression and in these the variability in the extent of depression was too great to allow mapping its time course. The depression could be quite obvious. For example, in one *T. crocea*, stimulated at one shock each five seconds, the first DOSP following a single HP averaged only 61% of the amplitude of the DOSP preceding the HP (s.e. = 5%, n = 28). The probability of this reduction occurring by chance is extremely low ($P < 0.005$). But this was an exceptional case and more usually depression was absent or too small to be readily detectable.

Depression following spontaneous tentacle activity

Distal tentacles show several kinds of spontaneous activity including oral or aboral movements of one or a few tentacles, simultaneous outward flaring of all

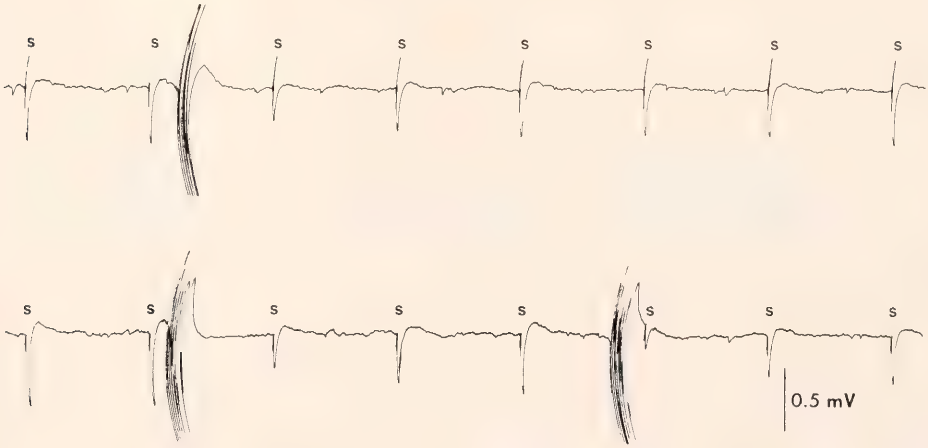

FIGURE 4. Depression of DOSP's following HP bursts. The upper and lower traces are from different parts of a long record during which the DOS was stimulated once each 10 seconds. Stimuli are marked by S in the figure. One HP burst occurs in the top trace, two in the lower trace. Note that DOSP's are depressed following these bursts. The records shown are a portion of those used to construct Figure 5A.

tentacles, and coordinated outward flaring beginning at one point on the tentacle crown and progressing around unidirectionally. The latter will be called a metachronal tentacle flare. Simultaneous outward flaring of all distal tentacles appears to be due to spontaneous firing of the DOS; it is associated with electrical potentials identical to evoked DOSP's. Evoked DOSP's following a spontaneous one are depressed, presumably because of the same defacilitation seen during series of evoked DOSP's.

There are electrical events associated with the other spontaneous tentacle movements as well. Activity in one or a few tentacles is recorded as small, irregular potential deflections; metachronal tentacle flares are recorded as large potentials, a bit smaller and slower than DOSP's and lacking the first DOSP component. The spontaneous tentacle movements and associated potentials are best seen in *T.*

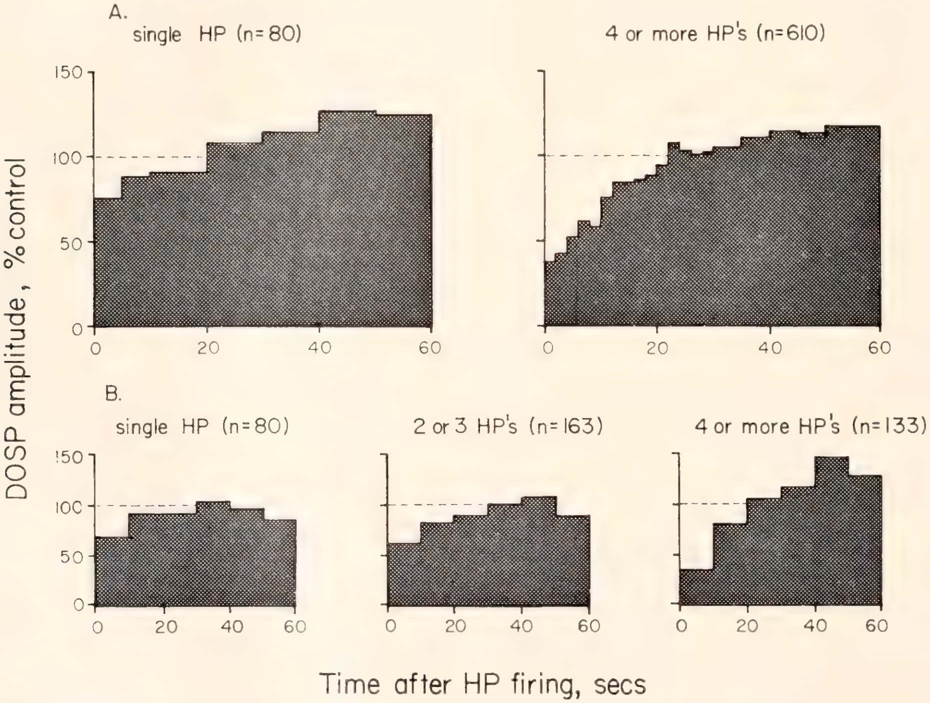

FIGURE 5. DOSP amplitude following HP activity. A is from the animal for which the longest series of records was collected. This animal produced only single HP's and long HP bursts. B is from another animal, one which produced bursts with 2 or 3 pulses as well as longer bursts and single HP's. The intervals between HP activity in these animals ranged from less than 5 seconds to more than a minute. Therefore the number of DOSP's in a given time block decreases with increasing time after HP activity; the longer the post-HP time the fewer the number of HP intervals long enough to contain that time. The bins in the histograms of A have been increased in width at longer post-HP times so that each bin contains approximately the same number of entries. Further explanation is given in the text.

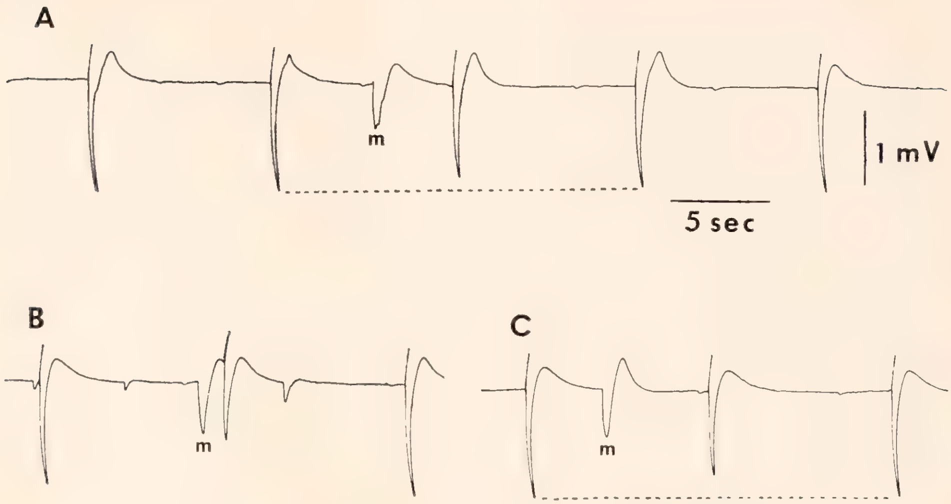

FIGURE 6. DOSP's following spontaneous distal tentacle activity. The events marked m are electrical potentials associated with sequential distal tentacle flaring in a metachronal wave. The dotted lines in A and C connect the DOSP peaks before and two after the spontaneous activity to emphasize the reduction in the DOSP immediately following the metachronal flare.

crocea which has an active proboscis and in which the electrical events are generally larger than in the other species.

DOSP's are depressed following aboral movements of scattered tentacles and particularly following metachronal tentacle flaring (Fig. 6). In the animal of Figure 6 metachronal flares occurred 13 times during periods of regular DOS stimulation at one shock each 10 seconds. The amplitudes of DOSP's immediately following metachronal flares averaged 91% of the DOSP's preceding the flares (s.e. = 2.5%, $P < 0.01$). Because spontaneous distal tentacle activity is of erratic occurrence and variable amplitude, no attempts have been made to quantify the time course of DOSP depression following this activity.

The most complete DOSP depression encountered occurred during defecation. In defecation the mouth slowly opens widely and rolls backwards over the proboscis, turning the proboscis inside out. In three animals, two specimens of *T. crocea* and one *T. spectabilis*, defecation occurred during a recording session without displacing the recording electrode. During the period when the mouth is open and inflected back over the proboscis, the second component of the DOSP is completely suppressed; DOSP's occur as only apparently normal first components.

DISCUSSION

The two components of DOSP's recorded from distal tentacles have different properties and are probably generated by different kinds of cells. The initial component is similar in shape and amplitude to DOSP's recorded elsewhere in the polyp. This component reflects activity in the conducting system, either electrogenesis by the conducting cells themselves or responses triggered in other cells by the conducted activity (see Ball and Case, 1973, for an example of separable conduction and electrogenesis). For reasons given above the graded second component of the DOSP is considered to be a muscle action potential, presumably generated by membranes of the epitheliomuscular cells which form the opening musculature of the distal tentacles. It was earlier suggested that the DOS is a neuronal conducting system (Josephson, 1965). The clear distinction between the first and second components of the DOSP (*e.g.*, Fig. 1) supports this view, at least it indicates that the DOS is not part of the same epithelial sheet which, in the vicinity of the distal tentacles, forms the opening musculature. Were the DOS an epithelial conducting system one would expect the recorded DOSP to gradually change when it entered the muscle area, perhaps becoming larger as the expanded membrane around the contractile processes became involved. Instead the DOSP is quite similar to that elsewhere but with a new component added after a distinct delay. While certainly not proving the identity of the DOS, the form of the recorded potentials is consistent with the idea that the initial DOS component is neuronal, the slower component from epitheliomuscular cells, and the delay between the two due to transmission processes between the conducting system and the muscle. In fact the delay between the first and second DOSP components is 9 to 20 msec which is sufficiently long that there may be several steps involved in the coupling between the two. This delay is longer than that between nerve net potentials and muscle action potentials in anemones (0–8 msec; Robson and Josephson, 1969; Pickens, 1969); about the same as the delay between conducting system pulses and evoked luminescence in the hydroid *Obelia* (Morin and Cooke, 1971) and considerably shorter than the delay between the onset of electrical activity and that of column contraction in hydra (100–150 msec; Josephson, 1967).

Several factors have been identified which selectively depress the second component of DOSP's: preceding DOS activity, HP firing, spontaneous distal tentacle activity including but not restricted to spontaneous DOSP's, defecation, and partial anesthetization with magnesium (for the last see Josephson, 1965). The recovery of tentacle responsiveness is slow, taking tens of seconds after HP firing and minutes after DOS activity. The constancy of the first DOSP component and the lability of the second indicates that factors affecting DOSP's act on the link between the two components, *i.e.*, they affect transmission processes between the conducting system and the muscle cells. There are several possible sites at which the DOSP reduction could be being effected; the membranes of the muscle cells (post-junctional depression), the terminals of the conducting system (pre-junctional depression) or unidentified elements interposed between the conducting system and the muscle cells. Some consequences of the first two possibilities as sites of depression will now be considered.

Depression of conducting system efficacy

Depression by excess magnesium and defacilitation can be readily interpreted as due to pre-junctional mechanisms. Excess magnesium characteristically blocks transmission at chemical synapses by reducing the amount of transmitter released by presynaptic terminals (*e.g.*, del Castillo and Engbaek, 1954), in turn resulting

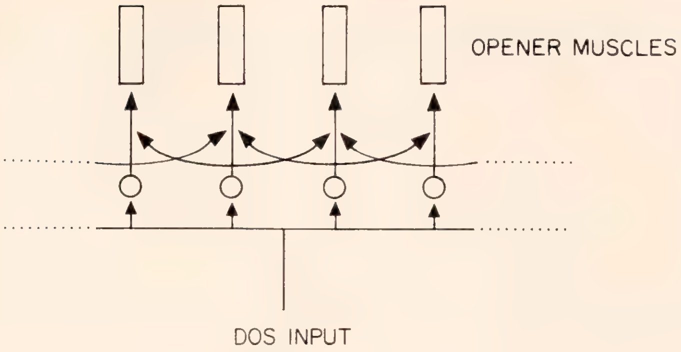

Figure 7. A scheme which could account for DOSP depression during repetitive DOS stimulation and following spontaneous distal tentacle activity on the basis of reduced efficacy of shared final terminals. The DOS synchronously activates a set of interconnected elements through polarized junctions. Spontaneous activity spreads laterally through the interconnected elements without exciting the main conducting pathway of the DOS.

in a smaller post-synaptic response. Although there may be both pre-junctional and post-junctional components in synaptic depression, a major factor is often depletion of available transmitter stores (*e.g.*, Thies, 1965). The obvious defacilitation of DOSP's could similarly be due to a reduced pool of available transmitter and hence less transmitter released to later stimuli of a series. The HP effects of DOSP's could also be due to reductions in the amount of transmitter released. The HP depression of DOSP's would then be an example of pre-synaptic inhibition. It is tempting to regard the DOSP reduction following spontaneous tentacle activity as due to the same defacilitation seen during repetitive DOS activation. If these are both pre-junctional effects it would require that spontaneous activity and DOS activation excite the opener muscles through the same final terminals. When activated by the DOS the tentacle opening appears synchronous; during spontaneous activity the tentacles can open sequentially, indicating rather slow lateral propagation. A scheme which would allow synchronous tentacle activation by the DOS and sequential activation during spontaneous activity is shown in Figure 7.

Depressed responsiveness of post-junctional membrane

Many of the features of DOSP depression could also be explained as due to the muscle membrane being partially refractory following activity. The time course of DOSP recovery would then reflect the time course of returning muscle responsiveness. Refractoriness following activity would readily account for the decline of DOSP's during repetitive stimulation and following spontaneous activity, but not for the reduction of DOSP's during magnesium anesthetization since this occurs without muscle activity.

There are at least two ways that HP firing could depress muscle responsiveness. The HP system may directly inhibit the muscle membrane by classical chemical inhibition. Alternatively the depression could again be related to post-firing

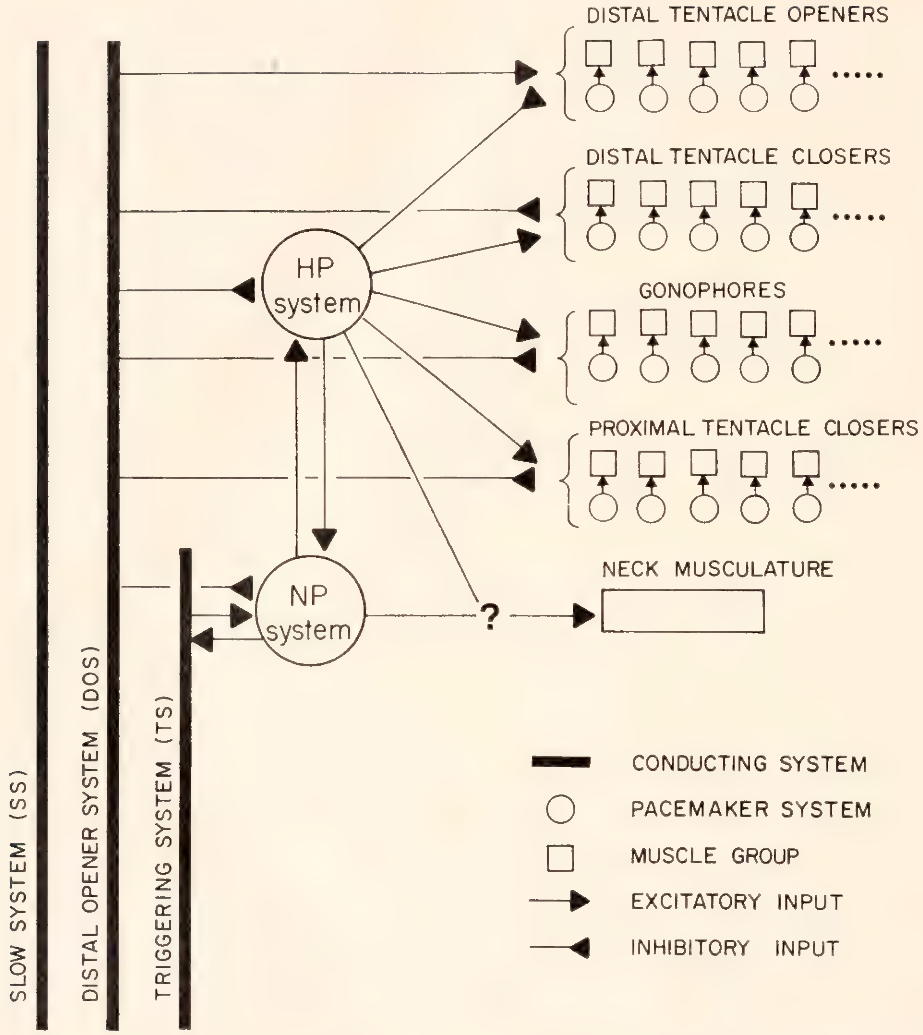
DISTAL TENTACLE OPENERS
DISTAL TENTACLE CLOSERS
GONOPHORES
PROXIMAL TENTACLE CLOSERS
NECK MUSCULATURE
HP system
NP system
?
SLOW SYSTEM (SS)
DISTAL OPENER SYSTEM (DOS)
TRIGGERING SYSTEM (TS)
CONDUCTING SYSTEM
PACEMAKER SYSTEM
MUSCLE GROUP
EXCITATORY INPUT
INHIBITORY INPUT

FIGURE 8. The interactions between conducting systems, pacemaker systems and effectors in a *Tubularia* polyp. This figure is based on one published earlier (Josephson and Uhrich, 1969) with the addition of gonophore interactions from unpublished observations of N. B. Rushforth and the inhibition of distal tentacle openers discussed in the text.

refractoriness. HP firing initiates weak tentacle closing, but this does not preclude the possibility that the HP system simultaneously activates the opener muscles and the closing seen is a balance between fully active closer musculature and weaker or only partially-activated opener musculature. It should be stated that there is no evidence for opener muscle activation during HP firing. DOSP's are not recorded from the polyp or stalk during HP firing, indicating that the conducting system is not excited. The muscle action potentials could be obscured by the usually larger HP's, but in some recordings from *T. crocea* the second DOSP component was larger than HP's and should have been seen during HP firing were it present.

So little is known about the mechanisms of mouth opening and proboscis eversion which are part of defecation that it seems fruitless to try to assign a prejunctional or a post-junctional site to the DOSP depression during defecation. Certainly the circular muscles around the mouth must relax; if the longitudinal muscles of the distal tentacles are involved in mouth opening or proboscis eversion is just not known.

The above discussion is not meant to imply that all factors affecting DOSP amplitude operate on the conducting system nor that all operate post-junctionally on the muscle membrane. These speculations do emphasize that there are a number of mechanisms which could account for the observed results and they indicate that with available knowledge it is not possible to distinguish which of the many possibilities are correct.

Reciprocal inhibition in Tubularia

From a behavioral viewpoint perhaps the most interesting of the interactions considered is the depression of DOSP's following HP firing. No matter what the mechanism, the net effect is inhibition of tentacle flaring by the HP system. The HP system and the DOS are antagonistic in their effects on the distal tentacles; the former initiating closing and the latter opening of the tentacles. The two systems are also mutually inhibitory although in an unusual way. Activity in the DOS inhibits pacemaker firing by the HP system (Josephson and Uhrich, 1969; Josephson and Rushforth, 1973), and HP activity inhibits the tentacle movements which are a consequence of the DOS activity. These interactions and others previously identified in *Tubularia* are summarized in Figure 8. This diagram emphasizes the complexity of behavioral coordination in *Tubularia*, a complexity which extends to the activation of individual muscle groups.

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SUMMARY

1. Activating the distal opener system (DOS), one of the conducting systems of the hydroid *Tubularia*, causes synchronous opening of the distal tentacles. Simultaneously recorded potentials (DOS pulses = DOSP's) from the vicinity of the responding muscles have two components: (a) an initial, short, all-or-nothing pulse reflecting activity in the conducting system, and, after a brief delay; (b) a

second, slower potential thought to be a muscle action potential because its amplitude varies monotonically with that of tentacle movement. The amplitude of the second component is here used as a measure of the intensity of muscle activation.

2. The second DOSP component is depressed by preceding DOS activity (defacilitation); by spontaneous tentacle movement including but not restricted to spontaneous DOS firing; by firing of the HP sytsem, one of the pacemaker systems in the polyp; and during the mouth opening associated with defecation. In all instances the first DOSP component is unchanged, indicating that the depression is of transmission processes between the conducting system generating the first component and the muscle membrane producing the second component. The recovery from depression is slow, taking tens of seconds following HP activity and minutes after DOS firing.

3. The HP system and the DOS are mutually inhibitory. Previous work has shown that activity in the DOS inhibits HP firing; here it was found that HP firing inhibits the tentacle response which is a consequence of DOS activity. The depression following spontaneous tentacle movements suggests that spontaneous activity and DOS firing may excite the muscles through a common final pathway.

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WATER TRANSPORT RATES OF THE TUNICATE *CIONA INTESTINALIS*[1]

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The interaction of suspension feeders with the environment depends primarily on the ability of these animals to transport sea water through their bodies. Although the determination of transport rates has been actively pursued by many investigators (Jørgensen, 1966), controversy exists over the accuracy and relevancy of the value produced by the use of a given method (Edmondson, 1966). The extreme rates, or the approximate average rate, are difficult to establish satisfactorily for tunicates due to experimental problems in handling the biological materials (*e.g.*, Hecht, 1916; Hoyle, 1953; Carlisle, 1966). Water transport rates of the tunicates *Styela clava* and *Ascidiella aspersa* were determined by different methods in static and in running sea water (Holmes, 1973). Even though the measured rates span a wide range, experiments with static sea water appear to yield lower transport rates than do those with running water. To establish sea water transport values for *Ciona intestinalis*, we are reporting transport rate studies using a variety of techniques. The average rates, or the extreme rates, are useful for determining the characteristics of sea water transport in suspension feeders and the relation of transport to tunicate physiology.

MATERIALS

The holding tanks of the New England Aquarium provide abundant specimens of a local species of tunicate, *Ciona intestinalis*. The freshly filtered sea water in which the experimental materials were maintained had a temperature of $16.0 \pm 0.2°$ C, an average salinity of $30.0 \pm 0.5‰$ (~ 0.51 M NaCl), and an average pH of 7.8 ± 0.3 at the time of experimentation. All specimens were kept in flow-through aquaria.

The 2-Methylquinoline (Quinaldine, Eastman Organic, practical grade) was used as anaesthetic to facilitate attachment of the tunicates to Pyrex tubes (≤ 6 mm O.D.) (Kustin, Ladd and McLeod, in preparation). The dye selected was a blue or green food color (Durkee's Food Color). The solvents for this dye, water and propylene glycol, were removed by freeze drying. The dye was then redissolved in sea water. At dye concentrations of approximately 0.01 ml original dye per 25.0 ml sea water, and/or high transport rates, *Ciona* was observed to react to the dye by squirting and ceasing to transport sea water.

In some experiments sea water-dye solution was supplied to the specimen with a Sage Variable Speed Tubing Pump, Model 375A. The Sage pump was calibrated by measuring the volume delivered to a clean dry graduated cylinder in a known time interval.

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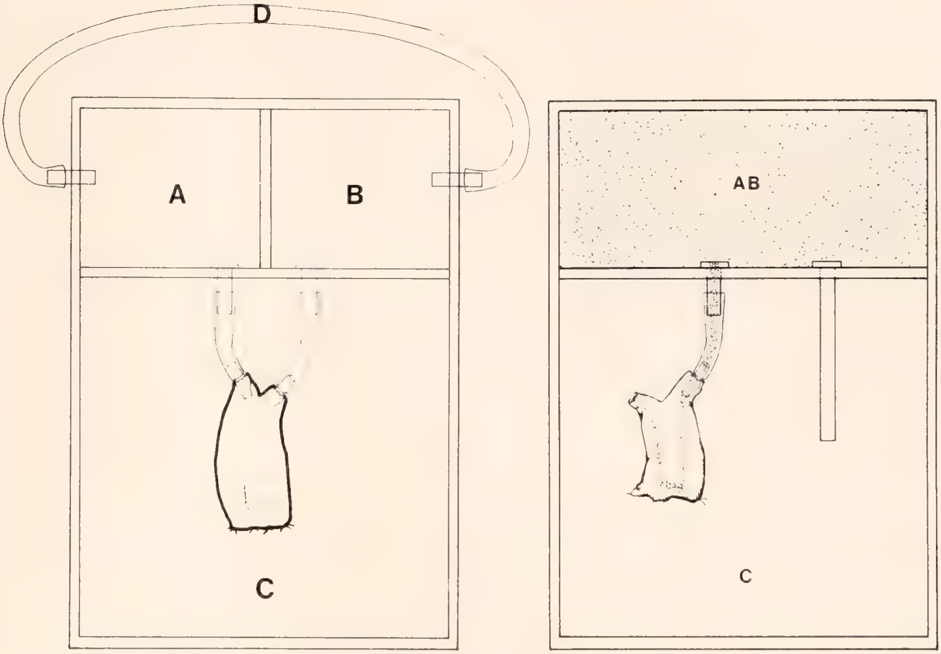

FIGURE 1. Overhead views of systems for determining the rate of sea water transport by the tunicate using the transfer of dyed sea water solution from compartment to compartment. The schematically rendered tunicate in the diagram shows the position of the attached specimen with respect to each compartment. *Ciona* which were used for experimental purposes lay horizontally, supported by the bottom of the container. *Left* shows *Ciona* attached by both siphons to Pyrex tubes with tygon tubing. Compartment A is attached to incurrent siphon; compartment B is attached to excurrent siphon; compartment C contains the body; tube D maintains equal levels. *Right* shows *Ciona* attached by incurrent siphon only to compartment AB. Compartment C contains the body. Plume of dye emitted by tunicate is schematically rendered. Return tube prevents the mixing of AB and C.

Dunaliella euchlora was grown at room temperature under fluorescent light banks in one gallon bottles in supplemented sea water type *f* (Guillard and Ryther, 1962). Stock cultures were similarly maintained sterilely at 19° C in 125 ml Erlenmeyer flasks. Algae were concentrated by centrifugation at 2500 rpm and resuspended in fresh sea water.

METHODS AND RESULTS

Volume methods

Specimens were attached to a partitioned chamber (FIG. 1, left, with connecting tube D absent). A typical rate of transport for a 4.0 g wet weight specimen was 180 ml hr^{-1} (or 45 ml/hr/g wet weight) determined by measuring the volume of overflow from Chamber B in 30 minutes. Careful observation showed that even under these conditions surface tension effects generated a pressure head approximately 1 mm between Chambers A and B.

Connecting tube D (Fig. 1, left) was added to eliminate the pressure head and a blue non-toxic dye was introduced into Chamber A. The rate was determined by monitoring the change in the absorbance of the solutions in Chambers A and B. One milliliter samples were removed simultaneously from the chambers at approximately six-hour intervals. Sea water was then carefully removed from Chamber C to keep all levels equal. Since the solution was pumped circularly the following treatment was used to calculate the flow rate produced by the tunicate.

In Chamber A, m_A is the moles of dye, C_A is the concentration of dye and V_A is the volume of sea water; similar terms, but with subscript B, refer to Chamber B. If the volume of solution transferred between chambers is ΔV, then the instantaneous rate of change of dye in Chamber A is

$$dm_A/dt = (d(\Delta V)/dt)\ (C_B - C_A) \tag{1}$$

assuming mixing in Chamber A. The total number of moles, M, of dye is conserved; therefore,

$$M = m_A + m_B \tag{2}$$

Also, the absorbance, A, of the solution in Chamber A is given by

$$A_A = alC_A \tag{3}$$

where a is the molar absorptivity coefficient, and l is the pathlength through the dye solution. Insertion of (2) and (3) into (1), rearrangement, and integration over the limits $m_A = m_A{}^0$ at time, $t = 0$ and $m_A = m_A$ at time, $t = t$ assuming constant flow so that $d(\Delta V)/dt = u =$ constant, and $V_A = V_B$ yields

$$ln\left(\frac{A_B - A_A}{A_B{}^0 - A_A{}^0}\right) = -2\left(\frac{u}{V_A}\right)t \tag{4}$$

The solutions in Chambers A and B were gently stirred to assure uniformity as infrequently as possible, since the tunicates often responded to stirring by diminishing transport. In a typical experiment, a 2.3 g specimen had an average rate of (5 ± 0.5) ml/hr/g wet weight, which appeared to be a rather low value.

Stress on the tunicate is relieved by reducing the number of attachments. Accordingly, only the incurrent siphon was connected (Fig. 1, right), the partition between Chambers A and B was removed, and the specimen transported sea water from Chamber AB into C. A return tube between AB and C kept the system at zero hydrostatic head. The samples for absorbance readings were taken approximately every 30 minutes for up to four hours, and analyzed according to equation (4). The results are presented in Table I.

In a further refinement, two specimens of *Ciona intestinalis* of approximately the same weight were attached to plastic bags partially filled with sea water. The bags were filled so as to exclude air bubbles, stoppered, weighed and allowed to equilibrate in a sea water bath. Under these conditions the pressure inside the bag should equal that outside. The specimens of *Ciona intestinalis* were anaesthetized and a Pyrex tube inserted in one siphon. They were then transferred to the sea water bath, allowed to revive and carefully connected to the bags so as not to

TABLE I

Transport rates for Ciona intestinalis *determined by volume flow. Column headings refer to the following: m, wet mass* Ciona; V_{AB}*, volume of sea water in compartment AB;* V_C*, volume of sea water in compartment C; u, transport rate; V′,* $(V_C + V_{AB})/V_C V_{AB}$.

m	V_{AB}	V_C	u, V'	u	u/m
g	*ml*	*ml*	hr^{-1}	$ml\ hr^{-1}$	$ml\ hr^{-1}g^{-1}$
1.3	78	1035	0.138 ± 0.006	10.0 ± 0.4	7.7 ± 0.3
3.0*	260	890	0.32 ± 0.01	65 ± 2	21.7 ± 0.8
3.0*	260	890	0.742 ± 0.006	149 ± 1	49.8 ± 0.4
3.0*	260	890	0.418 ± 0.006	84 ± 1	28.0 ± 0.4
3.0**	260	890	0.67 ± 0.34	134 ± 73	45 ± 24
2.5	260	1000	0.262 ± 0.006	54 ± 1	21.6 ± 0.5
2.1	78	1035	0.108 ± 0.009	7.8 ± 0.7	3.7 ± 0.3
2.1	260	1000	0.047 ± 0.004	9.6 ± 0.8	4.6 ± 0.4

* Same *Ciona* measurements over different 2 hour time periods in same day.
** *Ciona* as in * but measurement over 4 hours on next day.

change the mass of the system. One *Ciona* was connected via the incurrent siphon, the other via the excurrent siphon. After six hours the bags were carefully detached, stoppered and weighed. In this experiment a 2.2 g specimen transported in 71 g of sea water in 6 hours and a 2.5 g specimen transported out 64 g in the same period, yielding an average of 5 ml/hr/g wet weight. Volume methods appear to give low rates. Possible causes could be cilia impairment due to insertion of the tubes and/or stress. Therefore, the clearance technique was also used (Fox, Sverdrup and Cunningham, 1937).

Clearance method

Specimens were fed cultures of the green marine flagellate algae *Dunaliella euchlora*. The average size of *Dunaliella* was $4.6 \pm 0.9 \times 10^{-4}$ cm as determined by microscopical sizing. Twenty-four hours prior to experimentation, the tunicates were placed in aerated beakers containing known volumes of sea water. A 0.1–1.0 ml sample of *Dunaliella*, cell concentration $1\text{–}5 \times 10^8$ cells ml^{-1}, was then added. Aeration provided stirring, which, in addition to the motion of the algae,

TABLE II

Effect of time on cell counts with mixing by aeration. Organism is Dunaliella euchlora *in 100 ml sea water. Column headings refer to the following: t, time; N, cell counts.*

t	N
min	$cell\ ml^{-1} \times 10^{-5}$
0	1.14 ± 0.05
20	1.3 ± 0.8
40	1.20 ± 0.03
80	1.15 ± 0.07
mean	1.20 ± 0.07

keeps their distribution uniform and counteracts the action of gravity. A control experiment, consisting of an aerated beaker containing only sea water and *Dunaliella*, was sampled and cell counts taken to see whether gravitationally induced sedimentation was a factor that had to be taken into account. The data shown in Table II show that a uniform distribution of cells is maintained, within experimental error.

Samples were removed for counting at periodic intervals over a span of two hours to determine the rate of cell removal. Cell counts were made on a Wild microscope at 10 × 40 power using an American Optical Neubauer hematocrit.

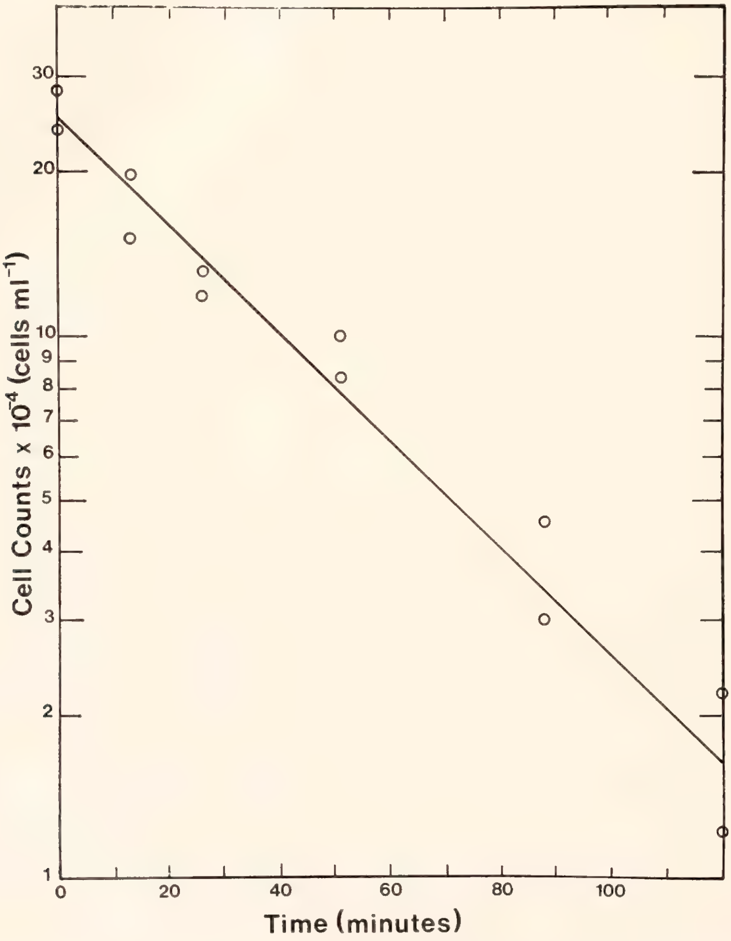

FIGURE 2. A semilog plot showing the relationship between cell counts per unit volume and time used in determining the rate of transport by particle clearance; experimental data: mass *Ciona*, 1.6 g; volume sea water, 100 ml; initial cell/ml $(2.47 \pm 0.04) \times 10^5$; u/V, 1.32 ± 0.12 hr^{-1}; u, 132 ± 12 ml/hr.

TABLE III

Transport rates for Ciona intestinalis *determined by clearance. Column headings refer to the following: m, wet mass* Ciona; *N, initial cell/ml; V, volume sea water; u, transport rate. Samples with same mass are same* Ciona *at different times.*

Day	m	N	V	u/V	u	u/m
	g	$cell\ ml^{-1} \times 10^{-5}$	ml	hr^{-1}	$ml\ hr^{-1}$	$ml\ hr^{-1}g^{-1}$
0	3.0	14.9 ± 0.8	250	2.2 ± 0.3	555 ± 70	185 ± 25
	1.5	13.7 ± 0.7	100	1.6 ± 0.2	160 ± 17	106 ± 18
	1.1	13.1 ± 0.4	100	1.02 ± 0.06	102 ± 6	92 ± 13
7	1.6	1.42 ± 0.04	100	1.3 ± 0.1	132 ± 12	82 ± 12
	1.6	2.47 ± 0.04	100	1.3 ± 0.1	132 ± 12	82 ± 12
	1.6	97 ± 3	100	0.82 ± 0.05	82 ± 5	51 ± 6
	2.4	2.5 ± 0.1	100	0.16 ± 0.01	16 ± 1	6.6 ± 0.7
	2.4	2.52 ± 0.06	100	0.11 ± 0.02	11 ± 2	5 ± 1
9	1.6	1.44 ± 0.07	100	0.11 ± 0.04	11 ± 4	7 ± 2
	1.6	23.2 ± 0.4	100	0.11 ± 0.02	11 ± 2	7 ± 1
	2.4	1.32 ± 0.09	100	0.78 ± 0.12	78 ± 12	32 ± 5
	2.4	22.5 ± 0.05	100	0.50 ± 0.04	50 ± 36	21 ± 15
	2.0	1.8 ± 0.1	100	0.28 ± 0.04	28 ± 6	14 ± 3
	2.0	25 ± 1	100	0.14 ± 0.04	14 ± 4	7 ± 2
15	1.6	10.9 ± 0.5	500	0.46 ± 0.10	231 ± 51	144 ± 32
	1.6	6.5 ± 0.2	500	0.40 ± 0.05	201 ± 27	125 ± 17
	2.4	10.7 ± 0.4	500	0.37 ± 0.04	183 ± 21	91 ± 10

The rate of removal was first-order (Fig. 2). The following first-order differential equation allows the transport rate to be calculated, assuming all cells passing into the tunicate are removed from the sea water.

$$-dC/dt = (u/V)C \tag{5}$$

In equation (5) C is cell concentration (cells/ml), u is the transport rate (ml/hr), and V is the volume (ml) of sea water in the beaker. The time constant u/V was determined by non-linear least squares analysis of equation (6) (Moore and Ziegler, 1960), the integrated form of equation (5), evaluated using the boundary condition $C = C_0$ at $t = 0$

$$C = C_0e^{-(u/V)t} \tag{6}$$

Transport rates based on this assumption are at least minimum values, since not all particles may be removed. To determine if the system was affected by cell concentration or volumes in which the specimens were kept, experiments were performed at low and high cell concentration (and volumes) on the same specimen.

In only one experiment (1.4×10^6 cells ml^{-1}) was any effect of cell concentration noted. This experiment terminated when the tunicate ejected a large mass of cells in a mucus-like matrix in the same manner as that previously described (Jørgensen and Goldberg, 1953) with respect to graphite uptake. In our case, however, the ejection was from the excurrent siphon. Otherwise the results (Table III) were independent of cell concentration. In a similar fashion, the volumes of sea water in which the animals were kept did not affect the rate of transport.

TABLE IV

Transport rates for Ciona intestinalis *determined by direct methods. Values of u represent instantaneous maxima. Column headings refer to the following: m, wet mass of* Ciona; *u, transport rate.*

m	u	u/m
g	ml hr^{-1}	ml hr^{-1}g^{-1}
1.8	95	53
1.6	70	44
1.8	60–90	33–50
1.5	20	15
1.8	140	78
2.0	110	55
1.8	70	39
1.5	125	83
1.6	5	3
2.4	5	2
2.0	5	2.5
1.6	21	13

Direct method

A direct method (Hamwi and Haskins, 1969) was modified to permit monitoring of the flow of a dyed sea water solution through a tunicate positioned in an upright manner. A variable flow Sage pump presented the solution at a right angle to the orifice of the incurrent siphon at a uniform rate (± 1%). The rate of flow of the dye solution was increased using the variable speed motor until it barely exceeded the rate at which the tunicate could remove it, whereupon the flow rate (assumed to be equal to that produced by the specimen) was recorded.

The specimens responded to the dye concentration. At high concentrations they would transport sea water until the solution reached the stomach; they then reacted by violently squirting out the dye. Consequently, the lowest possible dye concentration consistent with visibility ($A \sim 0.3$ at 635 nm) was used. With this technique, great variability in rates for a single specimen was noted. There were periods when the tunicates would not transport sea water; yet they appeared relaxed and normal. The values in Table IV are therefore instantaneous maxima and do not reflect prolonged transport.

DISCUSSION

Sea water transport in the tunicate serves the dual purposes of respiration and feeding. Neither of these functions requires continuous action. In fact, studies on the factors influencing rates of respiration have shown that, under stress, marine invertebrates may cease to transport sea water and respire anaerobically (Newell, 1973). Moreover, shutdown may follow exposure to low levels of oxygen (Holmes, 1973; Mangum and Van Winkle, 1973). Variability in transport has also been observed in our laboratory in connection with feeding and, of course, with squirting. Similar observations have been made for other ascidians; *e.g.*, *Phallusia mamillata* C. (Hoyle, 1953). Thus, intermittant transport and variability in the rate of transport mean that no single flow velocity is characteristic of a given species. Nor should a given method be expected to produce a single rate value. The average value does not represent a true instantaneous rate, but is useful for a number of applications.

The maximum rate found with acceptable volume methods, namely 28 ml/hr/g wet weight, is less than the previously reported value of 80 ml/hr/g wet weight (Goldberg, McBlair and Taylor, 1951). We experienced difficulty in attaching *Ciona,* necessitating the use of the anaesthetic quinaldine. No mention of this problem is made in the previous report; indeed, the specimen is shown in a figure therein pumping out of the incurrent siphon. Perhaps a larger specimen was attached in the earlier study, with less impairment to the ciliary action. Our experience indicates, however, that the adventitious occurrence of pressure heads is rarely avoided in a "constant level" device such as that previously described (Goldberg, *et al.,* 1951). We conclude that the earlier value represents a hydrostatically assisted transport rate.

Clearance rate studies would appear to be a more reliable indicator of realistic rates, although this method also has drawbacks (Hamwi and Haskins, 1969; Holmes, 1973). The maximum value in the range of rates determined in this study, 185 ml/hr/g wet weight, is less than the previously reported value of 230 ml/hr/g wet weight (Jørgensen, 1966), although it is greater than that recorded in the volume method studies. The average value is 62 ml/hr/g wet weight. The range of values determined by the direct method overlaps with both the volume methods (lower limit) and clearance (the maximum directly determined value being 83 ml/hr/g wet weight). The average value of clearance and direct methods is 50 ml/hr/g wet weight, which represents a realistic reference value for computational purposes.

It is interesting to compare this result with studies on the rate of vanadium isotope exchange in the tunicate *Ciona intestinalis* (Kustin, *et al.,* in preparation). For, despite the intermittancy in transport and the variability in rate, the intrinsic time constant for vanadium assimilation from sea water is constant; *i.e.,* it is independent of tunicate mass, for individual tunicates or groups of experimental materials. Suspension feeding and respiration are subject to control, accomplished through a variation in the rate of sea water transport. Nevertheless, even at its lowest level, the lowest transport time constant is greater than the vanadium assimilation time constant. In terms of the average value we find 50 ml/hr/g wet weight to be much larger than 6.5×10^{-2} ml/hr/g wet weight (Kustin, *et al.,* in preparation). Hence, water transport is the more rapid of the two consecutive processes. Once the sea water source of vanadium is forced into the tunicate's body, an essentially chemical extraction process, subject to little or no control, takes place, and does not show variability.

In addition to the above application, another example of the use that can be made of the average water transport rate data, is to estimate the physiological demand this activity places on the organism. Our visual inspection of the dyed sea water as it passes through the specimen shows a smoothly flowing column of fluid, which suggests that the Stokes-Hagen-Poiseuille laminar flow equation might be appropriate (Cole, 1962). This observation is supported by physiological studies of mucociliary action (Schlesinger, 1973), which suggest that the ciliary stroke produces a wave-like motion inducing smooth flow, and by a Reynolds number calculation on our data for *Ciona* (see below). By using fluid mechanics (Shames, 1962), the amount of mechanical, *i.e.*, pV (p is pressure), work done by the transport process can be related to the flow volume and the dimensions of the tube through which the volume of fluid flows.

We made a transparent photograph of *Ciona* transporting dyed sea water. The liquid column within the tunicate is visible through the translucent body. The photo was placed in an enlarger and brought to life size; the approximate dimensions of the column of dyed sea water were then measured. A 3.0 g specimen had dye solution in a U-shaped tube approximately 10 cm in length of narrowest diameter 0.08 cm and widest diameter 0.2 cm. (The plume of ejected fluid, clearly visible in the experimental aquarium and the photo, was approximately 0.15 cm wide and maintained this width for most of its length of 6–10 cm before dispersing). A diameter of 0.15 cm is therefore reasonable for the purpose of this estimation.

At an average rate of 50 ml/hr/g wet weight a 3.0 g specimen achieves a flow volume of 150 ml hr^{-1} or 4.2×10^{-2} cm^3 sec^{-1}. Sea water flowing at this rate in a 0.15 cm diameter pipe has a Reynolds number of ~36 and would therefore exhibit viscous laminar flow (Shames, 1962).

The internal pressure generated by the ciliary "pump" is equal to the viscous loss in the tube through which the fluid passes. (The losses at the tube ends are negligible in comparison). The internal pressure, Δp, is given by

$$p = 128\, qL\mu/\pi D^4 \tag{7}$$

where q is flow volume (4.2×10^{-2} cm^3 sec^{-1}), L is tube length exclusive of the "pump" (6.8 cm), μ is viscosity (1×10^{-2} dyne sec cm^{-2} for sea water at 20°), D is diameter (0.15 cm). The calculated internal pressure is 230 dyne cm^{-2} or ~2 mm H_2O, which may be compared to values of 3–4 mm H_2O for sponges (Parker, 1914; Bidder, 1923), and 2 mm H_2O for *Ascidia atra* (Hecht, 1923). The energy lost at the end of the excurrent siphon (*i.e.*, at the end of the tube) is 2.8 dyne cm^{-2}, which is negligible compared with losses elsewhere. Flow volumes in excess of the maximum value reported here lead to significantly higher, physiologically unreasonable internal pressures based on viscous losses. Moreover, the value of $\Delta p \sim 2$ mm H_2O shows the considerable influence of even the smallest external pressure.

SUMMARY

Sea water transport rates of the tunicate *Ciona intestinalis* were determined by measuring the volume of sea water transported through the specimen, and measuring the number of particles cleared by the specimen in a given time interval. The rate was also determined directly by matching the flow produced by the tunicate to that produced by a calibrated pump. *Ciona* transports sea water at variable rates; at times, it does not transport at all. The rate limits covering all techniques are: lower limit, 2.5 ml/hr/g wet weight and upper limit, 185 ml/hr/g wet

weight; the average value based on clearance and direct measurements is 50 ml/hr/g wet weight. Even at the lowest rate found, transport is rapid enough to ensure complete mixing between sea water and reaction or absorption sites in the pharyngeal chamber, alimentary tract or atrial chamber. We conclude that the rate controlling process for absorption of oxygen, vanadate ions, micro-organisms or organic detritus is not the rate of passage of the feeding current, but rather the rate of the intrinsic absorption process such as complex formation, ion exchange or adsorption.

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THE ACCESSORY CELL AND YOLK HALO OF THE OÖCYTE OF THE FRESHWATER TURBELLARIAN *HYDROLIMAX GRISEA* (PLATYHELMINTHES; PLAGIOSTOMIDAE)[1]

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Developing eggs are often associated with cells which, when numerous, form cell layers around the ovum, or, when single or few in number, appear to attach to the ovum in some way (Wilson, 1925). It is generally assumed that cells associated with eggs provide nutriment or act as nutrimental intermediaries to the growing oöcyte (Raven, 1961). In the turbellarian *Hydrolimax grisea* a single cell becomes intimately associated with the egg: this accessory cell completely surrounds the growing oöcyte and remains with the developing egg throughout its growth period (Newton, 1970). At the time of fertilization, the accessory cell remains in place around the egg—the sperm must penetrate the accessory cell before reaching the egg (Newton, 1970). Surrounding the oöcyte and its investing accessory cell in *Hydrolimax* is the yolk halo (Hyman, 1938; Newton, 1970). In histological preparations, the yolk halo consists of two layers, the inner of fine radial fibers and the outer of spherules. Hyman (1938; page 16) concluded the yolk halo to be ". . . a non-cellular halo presumably of nutritive nature." There is no evidence of utilization of the yolk halo material by the growing oöcyte or by the accessory cell (Newton, 1970).

Professor Ulric Dahlgren, of Princeton University, first observed the accessory cell and yolk halo of the oöcyte of *Hydrolimax* (Hyman, 1938). Opportunity for the present author to study some of Dahlgren's research notes and histological preparations of *Hydrolimax grisea* was made possible by a loan of the material from Dr. Ernst Kirsteur of the Department of Living Invertebrates of the American Museum of Natural History. The observations of Dahlgren (unpublished), Hyman (1938) and Newton (1970) are supplemented in the present report by phase-contrast and electron-microscopic studies of the accessory cell and yolk halo.

MATERIALS AND METHODS

The material for this study was obtained from sources previously reported (Newton, 1970). Wet-mount squash preparations of live material were studied and photographed with a Zeiss Photomicroscope using phase optics.

For electron microscopy, specimens of *Hydrolimax* were fixed whole, initially at room temperature or on ice, and subsequently diced with a razor blade in fixative on ice. The material was fixed for two hours in 3% glutaraldehyde in 0.1 M sodium cacodylate to which a trace of calcium chloride had been added, pH 7.1 (adjusted with HCl), followed by post-fixation in 2% osmium tetroxide, according to a procedure devised by D. P. Costello (personal communication). The material was dehydrated in a graded series of ethanol, passed through propylene oxide and embedded in Epon-Araldite. Sections were cut on a Sorvall Porter-Blum MT-2 ultra-microtome with glass or diamond knives and stained with 3% uranyl acetate (aqueous) alone or in combination with 0.5% lead citrate. The sectioned material was studied with a Zeiss 9S2 electron microscope.

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[1] Research performed in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Zoology, University of North Carolina at Chapel Hill, under the direction of Drs. Donald P. Costello and Catherine Henley.

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Observations

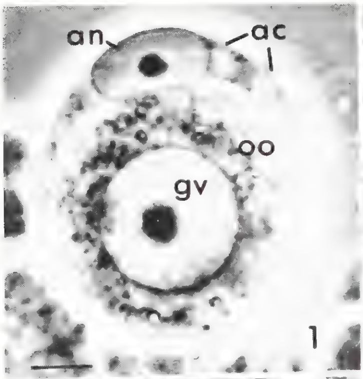
In the life cycle of *Hydrolimax,* female germ cells first appear in the parenchyma of worms collected in early fall of each year. Oögonia occur singly, or, rarely, in groups of three or more, not united by cell connectives or processes; they are free of association with other cells of the turbellarian parenchyma. Later many small, young oöcytes are found free of cell associations in squash preparations of living material.

The growing oöcyte becomes closely associated with cells of the parenchyma—the accessory parenchymal cells (Newton, 1970; his Figs. 3, 6). One of the accessory parenchymal cells becomes the accessory cell. Newton's Figure 7 shows an accessory cell fixed in the process of enveloping an oöcyte. By the mid-Fall of each year, the growing oöcytes of *H. grisea* have each been enveloped by an accessory cell. The chronology of the association of oöcyte and accessory cell can be correlated with cytological events of oögenesis: the accessory cell completely surrounds the young oöcyte after the dissolution of the chromosome bouquet (diplotene) prior to the extensive growth of the oöcyte.

Figure 1 is a phase-contrast micrograph of an oöcyte and its accessory cell. Although the phase halo in this micrograph makes it difficult to discern, the accessory cell has completely enveloped the oöcyte. Careful study through many planes of focus showed that the cytoplasm of the accessory cell is very thin on the side diametrically opposite the accessory cell nucleus. It may be significant to note that in squash preparations of living material accessory cells have not been observed *in the process* of enveloping oöcytes. The process of envelopment may be fairly rapid. The squashing of living turbellarians disrupts the parenchyma and apparently prevents normal accessory cell-oöcyte contact.

The oöcyte in Figure 2 is enveloped for the most part by a thin layer of accessory cell cytoplasm. In the vicinity of the accessory cell nucleus, the cytoplasm is thicker. Apparently the enveloping layer of accessory cell cytoplasm becomes attenuated by the relatively rapid growth of the oöcyte. Eventually, however, the accessory cell grows, for in later stages the layer of accessory cell cytoplasm surrounding the growing oöcyte thickens (Figs. 3, 4) (*cf.* Newton, 1970).

In squash preparations of live turbellarians collected in the late fall, the accessory cell, with its enveloped oöcyte, is surrounded by many accessory parenchymal cells (Fig. 3). The number of accessory parenchymal cells increases. By mid-Winter the accessory parenchymal cells have *apparently* been replaced by the spherules of the yolk halo (Fig. 4) (*cf.* Newton, 1970). The accessory parenchy-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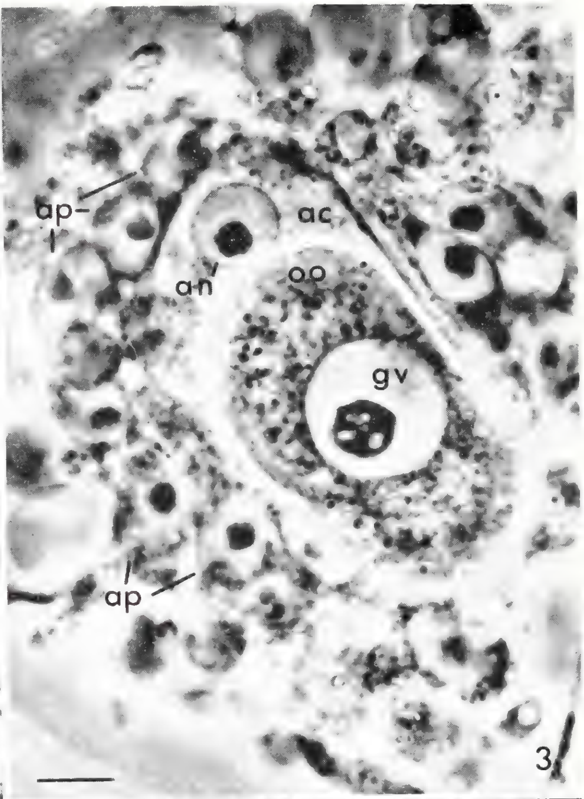
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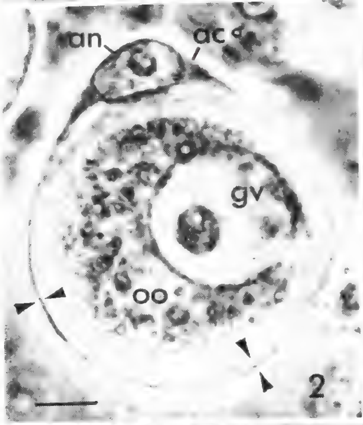
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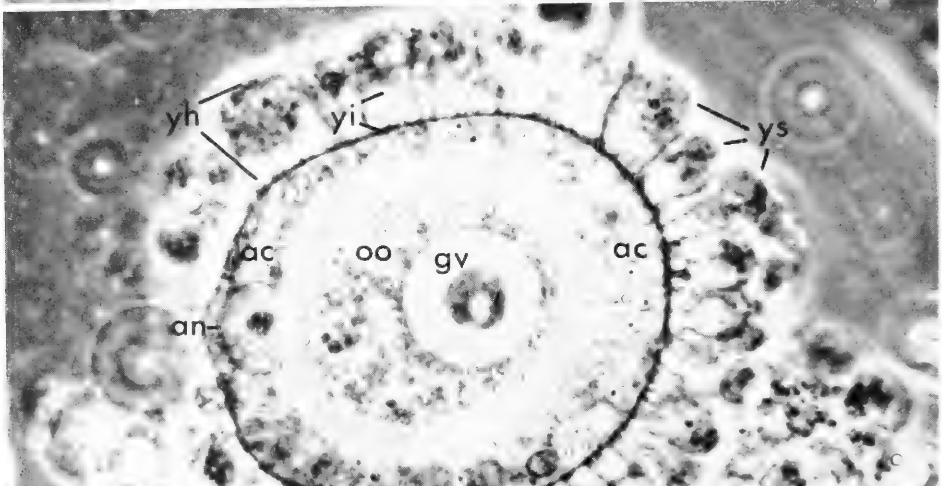
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mal cells, however, become transformed into the spherules of the outer portion of the yolk halo (Fig. 4).

The accessory cell is in close apposition to the oöcyte (Figs. 1–5). In some specimens fixed, sectioned and stained for light microscopy, a wide space exists between the inner cell boundary of the accessory cell and the surface of the oöcyte (Newton, 1970; his Figs. 8, 12). The space is an artifact of fixation caused by shrinkage of the oöcyte, for no space is seen between accessory cell and oöcyte in living material studied by phase optics. A shrinkage space exists between oöcyte and accessory cell in some specimens fixed and sectioned for electron microscopy. Generally, however, electron micrographs show the accessory cell to be separated from the oöcyte surface by a distance no greater than 0.2 μ (Fig. 5). Cytoplasmic processes from the inner surface of the accessory cell penetrate the oöcyte no deeper than 0.8 μ (Figs. 5, 6, 7). The cytoplasm of the accessory cell and the cytoplasm of the oöcyte do not appear to intermix. The membranes of the two cells remain intact. The tips of the accessory-cell projections often touch the surface of the oöcyte (Fig. 6) and prehaps fuse with the cell membrane of the oöcyte, as indicated in Figure 6. The space between oöcyte and accessory cell is empty in specimens prepared for light microscopy. In material prepared for electron microscopy amorphous particles of medium electron density are found between oöcyte and accessory cell (Figs. 5, 6, 7). Similar amorphous particles are located within membrane-bound vesicles of the accessory cell (Figs. 5, 6, 7). An open vesicle in Figure 6 suggests an interchange of amorphous particles between accessory cell and the intercellular space. In sections stained with uranyl acetate or with uranyl acetate in combination with lead citrate, electron-dense bodies of unknown significance are located on or immediately beneath the plasma membrane (Figs. 5, 7, and especially 6) of the oöcyte.

Sections through accessory cells show that the cytoplasm immediately beneath the outer and inner membranes is uniformly compact and generally devoid of organelles and inclusions (Figs. 6, 7). The layers of relatively homogeneous cytoplasm at the surfaces of the accessory cell are here called the outer cortex and the inner cortex. The cortices are approximately 730 Å thick. The cortical cytoplasms are granular, consisting of dark particles, approximately 100 Å or less, in a ground substance of medium electron density (Figs. 6, 7). Between the cortices are all manner of membranous elements and some granular components (Figs. 5–9). Mitochondria are the only distinctive organelles. Whorls of membrane, membranous cisternae of irregular shape containing wisps of electron-dense material, and endoplasmic reticulum are the major components of inter-cortical cytoplasm.

Microvilli arise from the outer surface of the accessory cell (Figs. 5, 7) and often project 3 to 4 μ into cells of the turbellarian parenchyma (Figs. 5, 8). The

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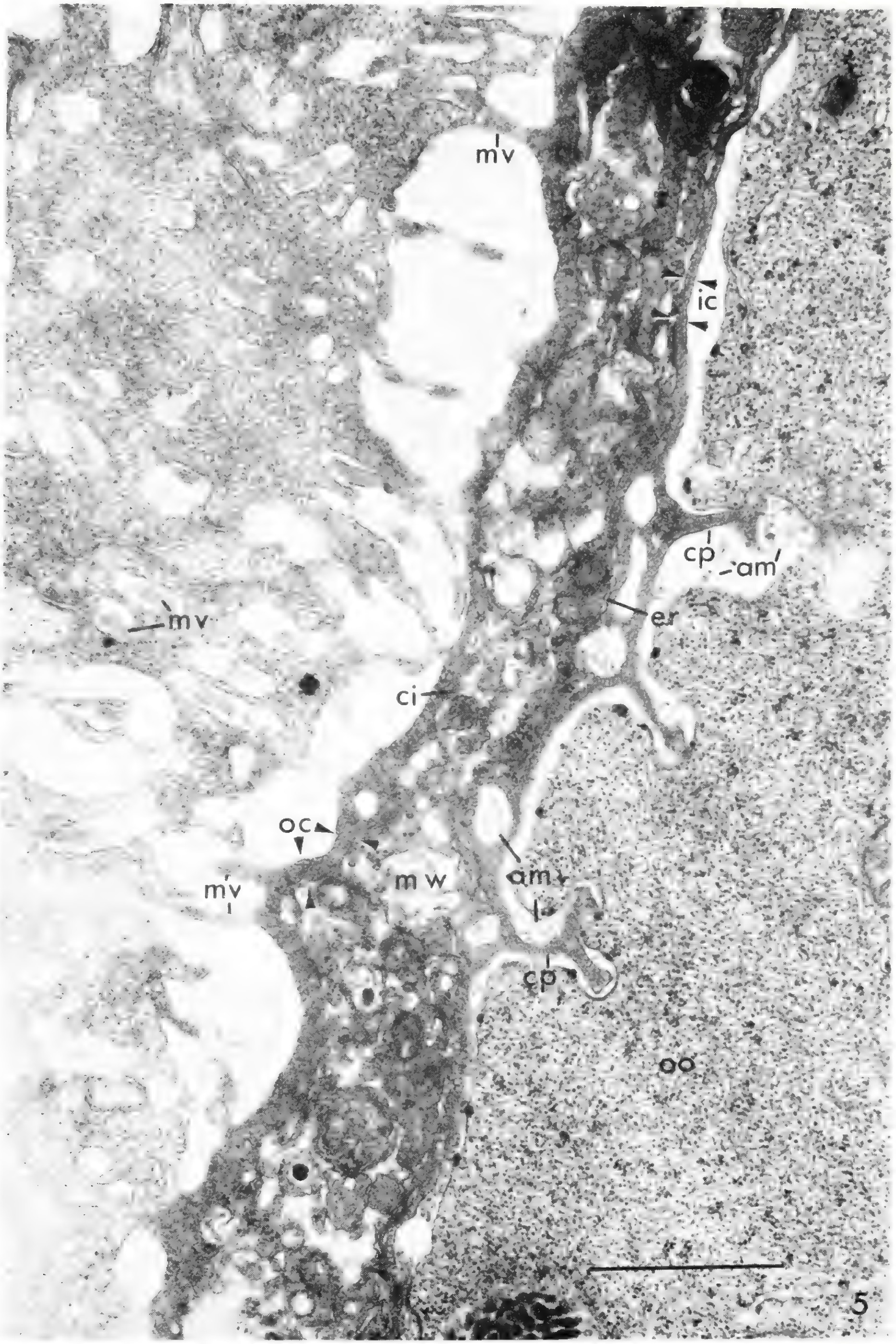
FIGURES 1–4. The events depicted in this plate occur over a five- to six-month period. The young oöcyte is surrounded by an accessory cell (Fig. 1) before oöcyte growth (Fig. 2). Accessory parenchymal cells become associated with the outer surface of the accessory cell (Fig. 3) and transform into the spherules of the outer portion of the yolk halo (Fig. 4); phase-contrast micrographs; Figures 1, 2, 4, 784×; Figure 3, 980×; ac, accessory cell; an, accessory cell nucleus; ap, accessory parenchymal cell; gv, germinal vesicle (nucleus) of oöcyte; oo, oöcyte; yh, yolk halo; yi, inner portion of yolk halo; ys, spherules of outer portion of yolk halo; scale = 10 μ.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microvilli of the accessory cell do not fuse with adjacent cells but occupy membrane-delimited canals within the cytoplasm of adjacent cells (Figs. 5, 8). The appearance by light microscopy of the inner portion of the yolk halo, consisting of fine radial fibers (Fig. 4) (Newton, 1970; his Fig. 17), is explained by the numerous microvilli which extend from the accessory cell. Electron-dense filaments are oriented longitudinally within microvilli (Figs. 7, 9); no filaments are observed in the projections from the inner surface of the accessory cell into the oöcyte (*cf.* Figs. 5, 6, 7). Four to eight filaments are observed in transverse profile of microvilli (Fig. 9).

Evidence for the transformation of accessory parenchymal cells into the outer layer of the yolk halo was first obtained by the present author from some of the slides of Dahlgren. In Dahlgren's material, fixed with hot Flemming's fluid and stained with an undesignated hematoxylin, a basophilic material is present in the spherules of the outer layer of the yolk halo; this material is not bounded by a nuclear membrane. Dahlgren suggested in his research notes that the nucleus of the accessory parenchymal cell may be degenerating in the process of transformation by the accessory parenchymal cell into a yolk-halo spherule. In material prepared for light microscopy in Newton's (1970) study, evidence for nuclear degeneration is lacking: no basophilia is seen in the yolk spherules. Newton (1970; his Fig. 12) suggested that the yolk halo may be secreted by the accessory parenchymal cell. The discrepancy between Dahlgren's observations and those of Newton's study is probably a result of the use of different methods of fixation and staining. Evidence that Dahlgren's interpretation is correct comes from electron micrographs of the outer layer of the yolk halo (Figs. 8, 10). The spherules of the yolk halo are composed of organelles, primarily vesicles, but also endoplasmic reticulum, a few scattered mitochondria, some microtubules, all bounded by a cell membrane, and without nuclei. After Feulgen-Fast Green the yolk halo spherules are Feulgen-negative. The vesicles (Fig. 10) are conspicuous because of the homogeneous, electron-dense material unevenly distributed within them. Transformation of accessory parenchymal cells into the spherules of the outer portion of the yolk halo remains an enigma: the function of the spherules has not been ascertained. Evidence for the utilization of the yolk-halo spherules by the oöcyte is lacking.

DISCUSSION

According to the system of classification of eggs proposed by Korschelt and Heider (1902; cited in Wilson, 1925; and Raven, 1961), the egg of *Hydrolimax* is of the nutrimental type and its accessory cell is a nurse cell. The accessory cell

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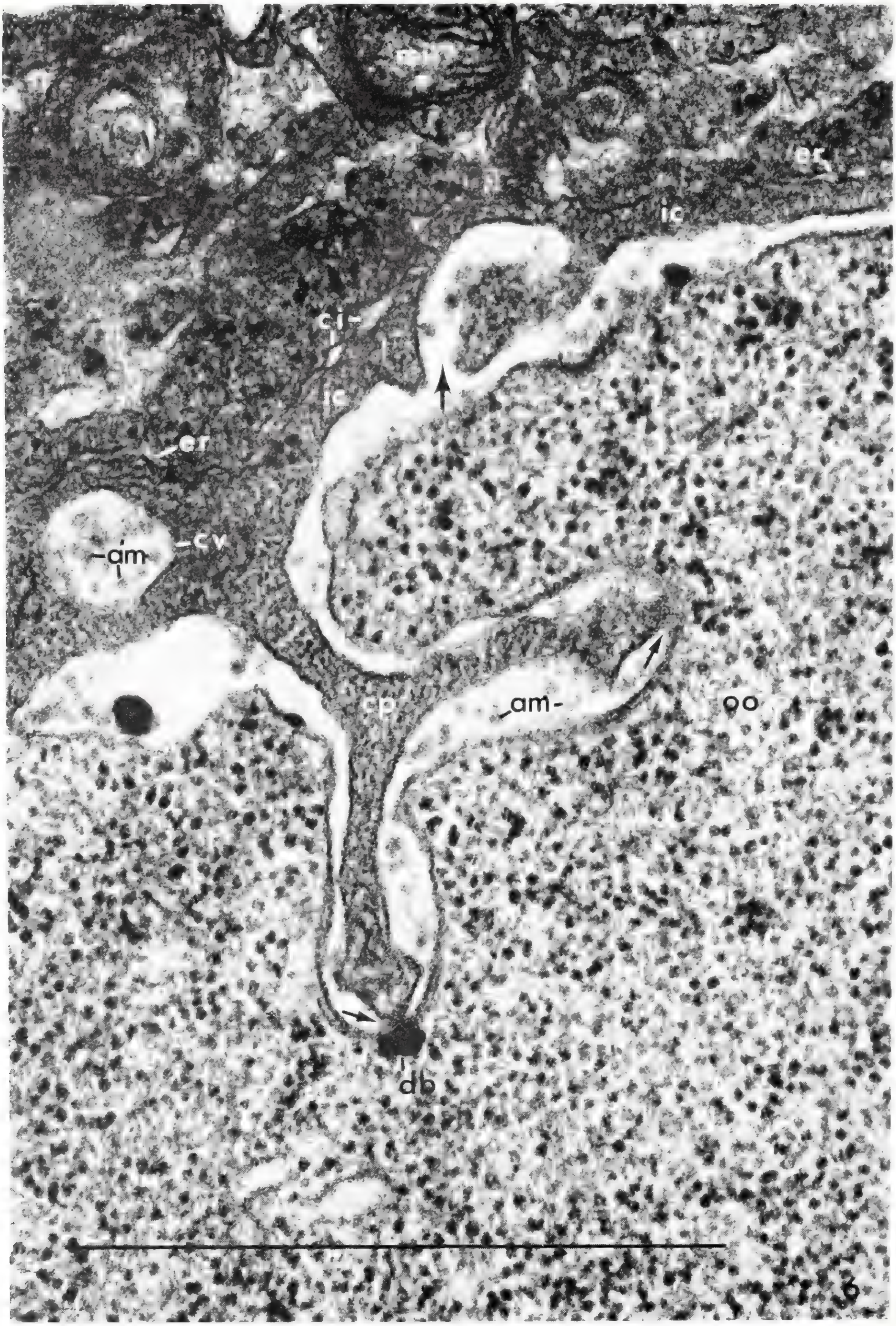
FIGURE 5. The relationship between accessory cell and oöcyte is marked by the cytoplasmic processes extending from the inner surface of the accessory cell into the developing egg. Microvilli extend from the outer surface of accessory cell into membrane delimited canals in cells of the turbellarian parenchyma; uranyl acetate; 26,871×; am, amorphous particles; ci, swollen cisternae of endoplasmic reticulum; cp, cytoplasmic process; er, endoplasmic reticulum; ic, inner cortex; mw, membranous whorl; mv, microvillus; oc, outer cortex; scale = 1 μ.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of *Hydrolimax* is unique among the plagiostomids (Hyman, 1938) and apparently unique among the animal phyla.

The relationship between accessory cell and oöcyte in *Hydrolimax* is one of close apposition of the cells involved, and of cytoplasmic projections into the oöplasm from the inner surface of the accessory cell. There is no evidence that the cytoplasms of the accessory cell and of the oöcyte intermingle. Gondos (1970) observed close apposition of cell membranes of germ cells and granulosa cells, and the presence of interdigitating cytoplasmic projections from granulosa cells into oöcytes, of young rabbits. In the human, "localized projections from both the oöcyte and the follicle cells interdigitate in the intercellular space and appear to reflect a constant active interchange between the oöcyte and its follicle wall" (Hertig and Adams, 1967; page 668). The extension of accessory cell processes into the oöcyte in *Hydrolimax* may likewise reflect an interchange between the two cells.

Microvilli extend from the outer surface of the accessory cell, into extracellular spaces or into membrane-delimited canals of adjacent parenchymal or oviducal cells. Since microvilli are generally present on cells which have absorption as a primary function (Bloom and Fawcett, 1968), the function of the outer surface of the accessory cell may be to absorb nutrient material for transport across the accessory cell to the growing oöcyte. The speculation is amenable to simple experimental analysis, as the oöcyte and its surrounding accessory cell are easily isolated from the parenchyma of young turbellarians.

The author is grateful to Dr. Donald P. Costello and Dr. Catherine Henley for advice during the course of this study and for helpful criticisms during the preparation of the manuscript. Mrs. Wilma Hanton is very much appreciated for her patient advice and help with electron microscopy. The work was aided by a grant to Dr. Costello and Dr. Henley from the National Institutes of Health, GM 15311.

SUMMARY

1. A single cell, the accessory cell, completely surrounds the growing oöcyte of *H. grisea*.

2. Cytoplasmic processes from the inner surface of the accessory cell extend into the oöcyte. The cytoplasms of the accessory cell and of the oöcyte do not intermix.

3. Microvilli extend from the outer surface of the accessory cell.

4. It is suggested that the accessory cell is a nurse cell which transports nutrient material to the growing oöcyte.

FIGURE 6. Small arrows indicate apparent contact of tips of cytoplasmic process with oöcyte membrane. Large arrow points to opening of a cytoplasmic vesicle of accessory cell into intercellular space; uranyl acetate, 91,140×; cv, cytoplasmic vesicle; db, dense body; mi, mitochondrion; scale = 1 μ.

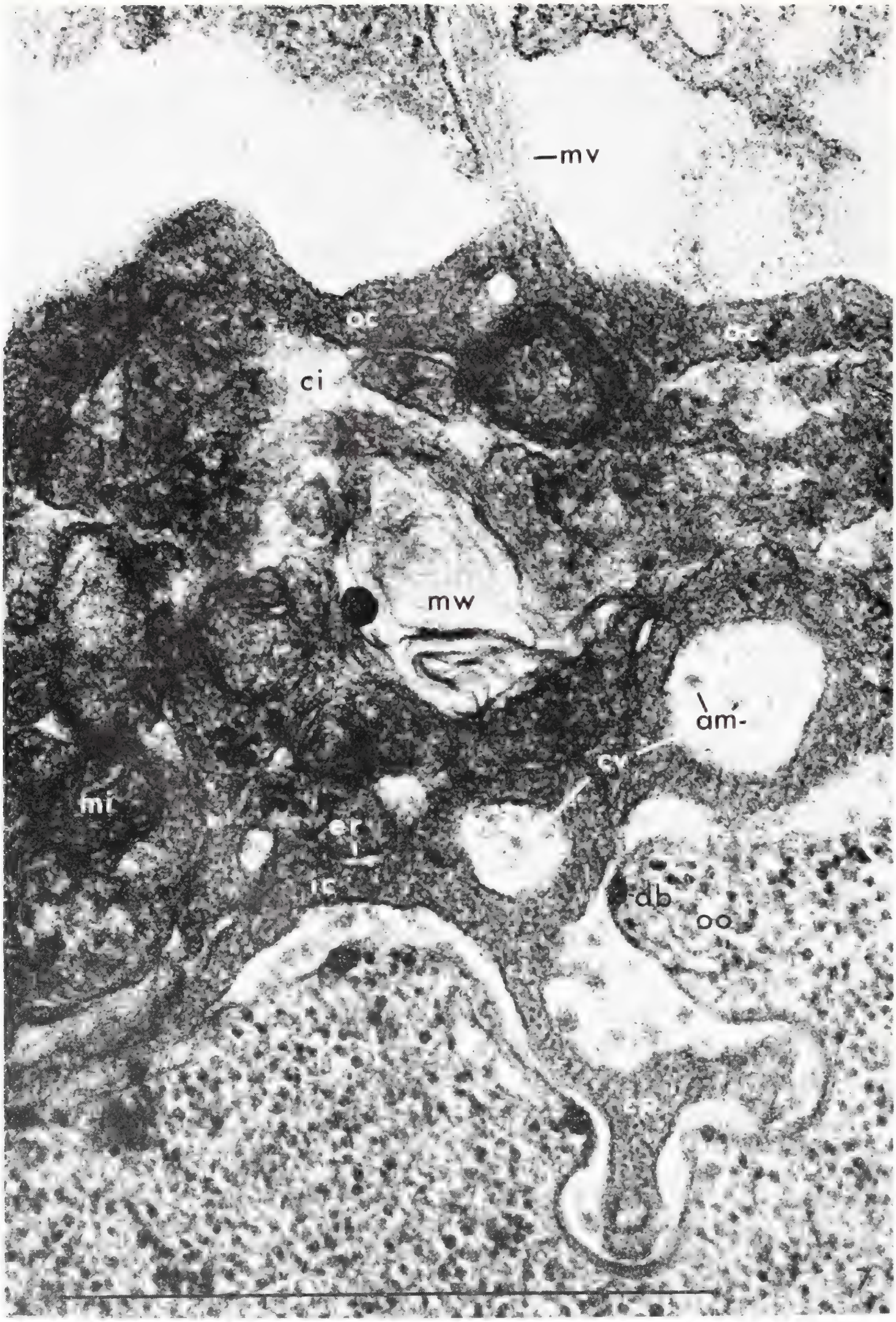
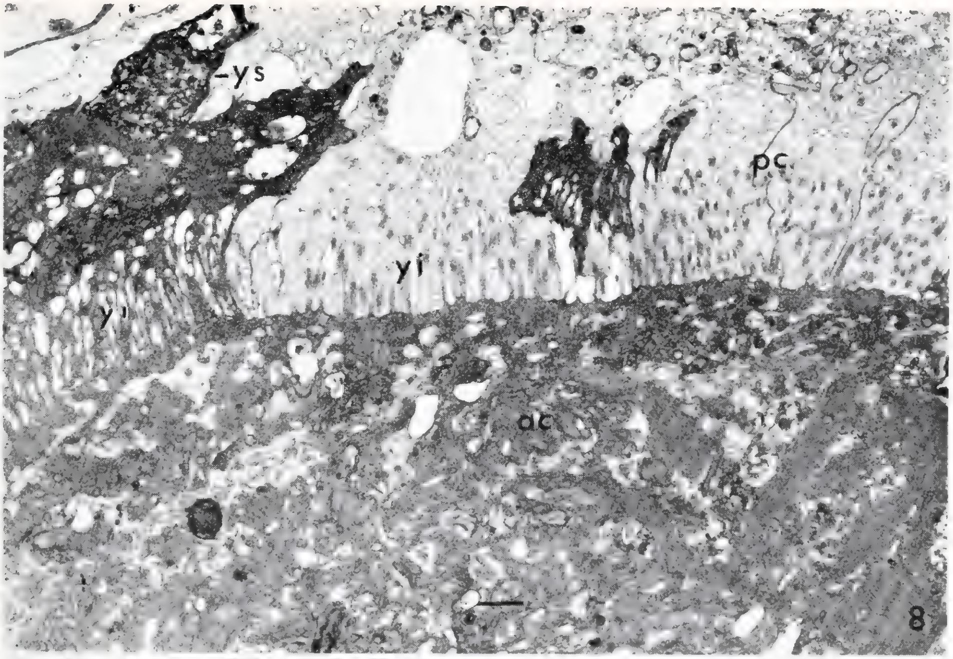
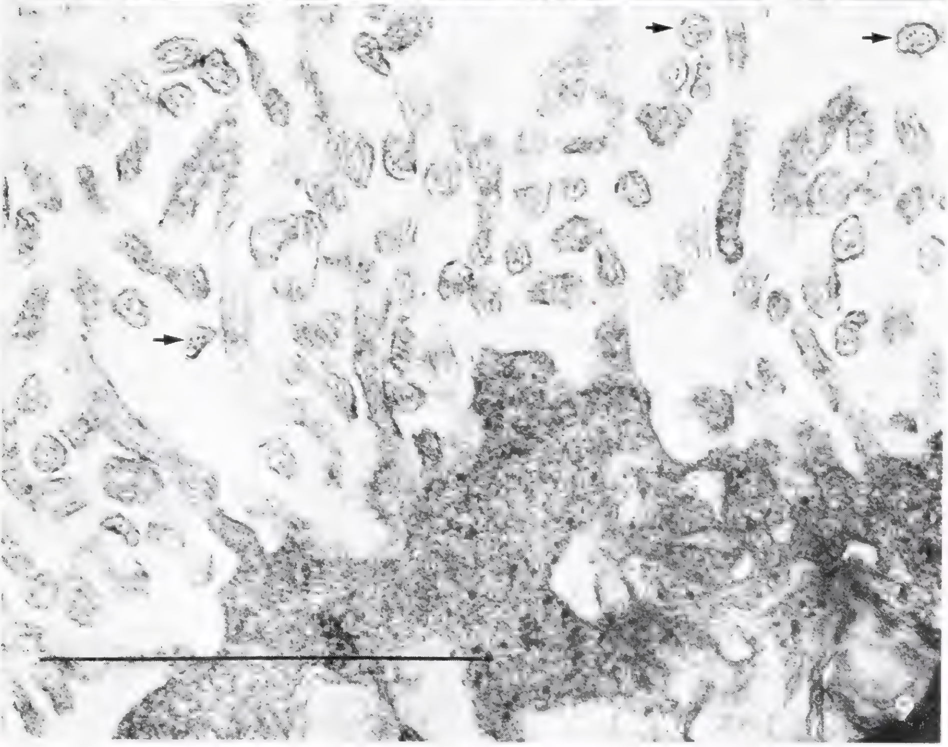


FIGURE 7. The ultrastructure shown in this electron micrograph quite clearly suggests the function of the accessory cell: absorption of nutriment for transport to the growing oöcyte. Microvilli absorb nutrients which are then transported or stored until required in the voluminous cisternae. Amorphous particles may represent processed material to be utilized by the egg; uranyl acetate, lead citrate: 91,140×; scale = 1 μ.





FIGURES 8 and 9. Many microvilli extend into parenchymal cells and remnants of accessory parenchymal cells, now changed to yolk spherules (Fig. 8). The microvilli in Figure 9 are free of enveloping cytoplasm. Arrows point to transverse sections which show distinct profiles of microvillar filaments; uranyl acetate; Figure 8, 5468×; Figure 9, 58,800×; scale = 1 μ.

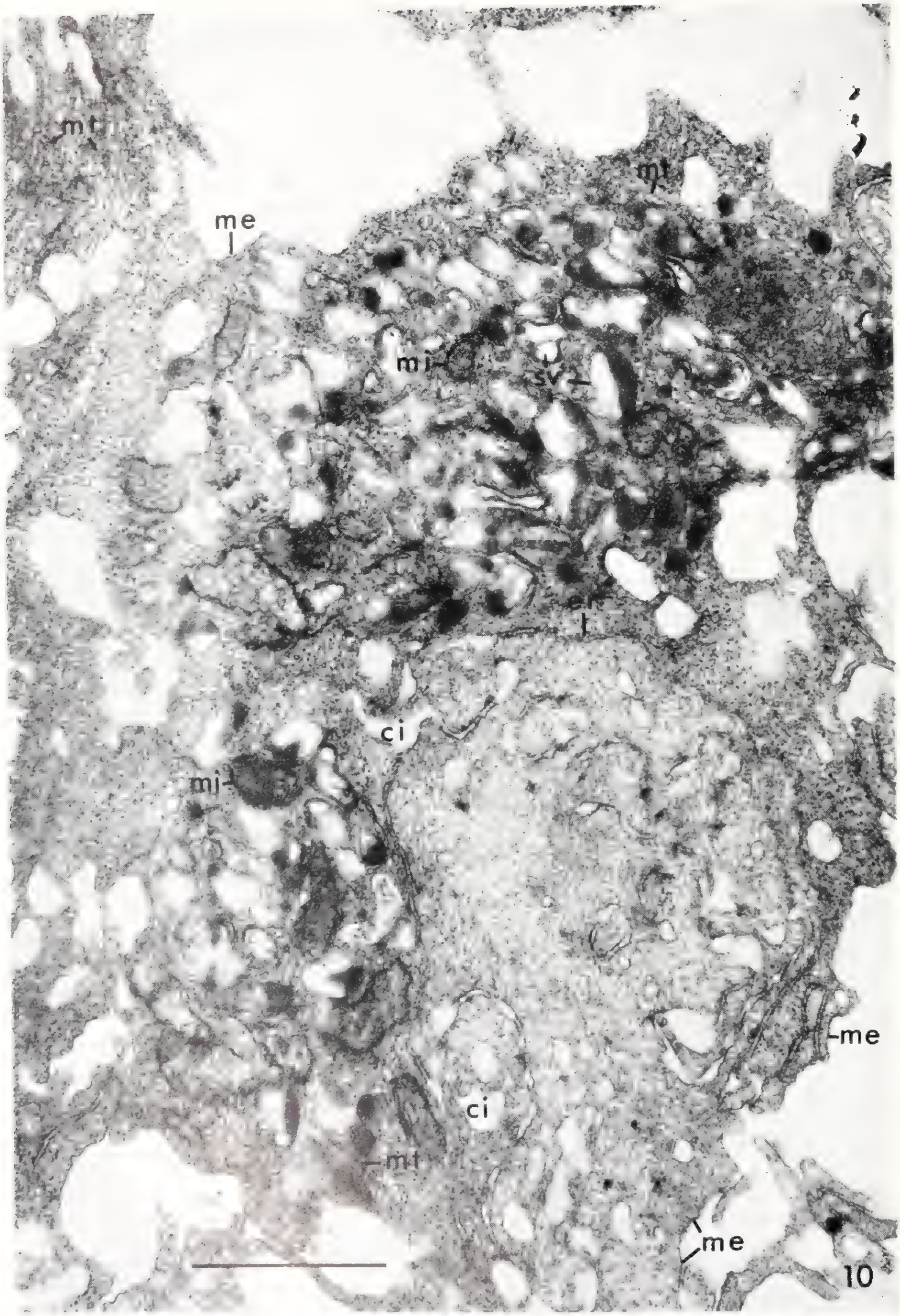

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FIGURE 10. A single yolk spherule is composed of cellular elements without nucleus. Linear densities at surface suggest that the yolk spherule is membrane bound; uranyl acetate; 26,871×; me, membrane; mt, microtuble; sv, spherule vesicle; scale = 1 μ.

5. The yolk halo consists of two layers, each of different origin. The inner layer is composed of the numerous microvilli which extend from the accessory cell into membrane-delimited canals within cells of the turbellarian parenchyma. The spherules of the outer portion of the yolk halo are derived from accessory parenchymal cells. The function of the yolk-halo spherules is unknown.

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Reference: *Biol. Bull.*, **147**: 630–640. (December, 1974)

MODIFICATION OF SEA ANEMONE BEHAVIOR BY SYMBIOTIC ZOOXANTHELLAE: PHOTOTAXIS

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Symbiosis between an animal host and a photosynthetic symbiont is unlikely unless the animal spends considerable time in the sun. So it is not surprising that a variety of invertebrates with unicellular endosymbiotic algae display positive phototaxis. Probably the first report of phototaxis in an algae-bearing invertebrate is Trembley's (1744) observation that green hydras gather on the lighted side of a glass vessel. Later investigations show, however, that other, non-symbiotic hydra species (Wilson, 1891) and green hydras which have lost their symbionts (Whitney, 1907) also respond positively to light. The acoel flatworms *Convoluta roscoffensis* and *C. convoluta* show phototactic behavior correlated with their usual habitats (Keeble, 1910). Again, the symbiotic algae are not essential to the response. The action spectrum for phototaxis does not correspond to that of chlorophyll, and light apparently is received by the carotenoid-containing eyespots of the animal. Even worms just a few hours old and still white respond phototactically. Vandermeulen, Davis, and Muscatine (1972) mention that *Placobranchus ianthobapsus,* a saccoglossan opisthobranch gastropod with functional chloroplasts derived from siphonaceous green algae, displays positive phototaxis even when chloroplast photosynthesis is effectively eliminated by chemical inhibition. In other cases, however, phototaxis appears to depend on the intact, functional symbiosis. The ciliate *Paramecium bursaria* with zoochlorellae shows positive phototaxis, and the action spectrum of the response corresponds to the absorption spectrum of chlorophyll (Engelmann, 1882). *P. bursaria* without algal symbionts does not show positive phototaxis (Siegel, 1960), nor do any of the other, non-symbiotic species of *Paramecium* show directed light responses (Gelber, 1956; Halldal, 1964).

Besides the green hydras, there are several other examples of tactic responses to light among algae-bearing cnidarians. Coral planulas containing zooxanthellae swim toward light; those without zooxanthellae display no light response (Kawaguti, 1941, 1944; Atoda, 1953). Zahl and McLaughlin (1959) report that *Condylactis* sp., a Caribbean sea anemone with zooxanthellae, moves out of direct sunlight into shade, but they did not establish whether the anemones respond positively to light of lower intensity. Specimens of *Condylactis* that are kept in the dark lose their symbionts and also lose their phototactic response. Cotte (1921, 1922) describes inconsistent phototactic behavior in *Anemonia sulcata,* a European sea anemone with zooxanthellae. Of the few other sea anemones in which phototaxis has been tested, all non-symbiotic species, most appear either indifferent or negative to light (*e.g.*, Hargitt, 1907; Parker, 1917; Ottaway, 1973).

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Most littoral anemones live in shaded habitats. However, the two algae-bearing sea anemones of the west coast of North America, *Anthopleura elegantissima* (Brandt, 1835) and *A. xanthogrammica* (Brandt, 1835), are unusual in distribution; most occupy rocks and tidepools in full sun. This unusual habit, apparently associated with the symbiotic relationship, led me to suspect that these anemones might show responses to light which were different from those of most actinians.

Anthopleura elegantissima is particularly suitable for studying possible relationships between behavior and symbiosis because the animals are hardy and abundant; exposed for collection at almost any low tide; mostly conveniently small (although solitary individuals may be as large as many large *A. xanthogrammica*); and reproduce asexually by dividing, forming distinct clonal beds with tens to hundreds of genetically identical individuals (see Francis, 1973). Clones can be found in light conditions grading from direct sunlight to deep shade, with the number of algal symbionts correspondingly graded. It is possible to introduce zooxanthellae into anemones that lack them (Trench, 1971c); and individuals with algae can be made to lose their symbionts, yet remain apparently active and healthy (see Methods). As part of a more extensive study on the symbiosis of *A. elegantissima* with zooxanthellae (Buchsbaum, 1968; Pearse, 1974), I have tested anemones of this species, with and without symbiotic algae, for evidence of phototactic behavior and have tried to discover whether their behavior and symbiosis might be related.

METHODS

Specimens of the sea anemone *Anthopleura elegantissima* with abundant symbiotic algae were collected from two rocky intertidal areas in central California. Specimens with few and no zooxanthellae were collected from intertidal rocks or cement pillars beneath the cannery buildings, Cannery Row, Monterey. Only golden-brown dinoflagellate zooxanthellae were present as algal symbionts in the anemones.

Tests for phototaxis

The sea anemones were tested for phototaxis in shallow seawater tables. Eight 112-cm Sylvania Lifeline fluorescent tubes were suspended 30 cm above the table; they were left on continuously during tests. Incident illumination at the center of the table was 700 foot-candles, as measured with a Photovolt 200 photometer, also used for intertidal light measurements. A continuous flow of sea water along the table maintained the temperature at 14° C ± 1° C. The water was approximately 4 cm deep and covered the anemones. A wooden board supported just above the surface of the water shadowed half of the experimental area. The total area was 22 cm across by 125 cm long.

At the beginning of each of the 19 experiments, 25–70 anemones were placed in the table under uniform illumination. After the anemones had all attached to the surface of the table, their positions were recorded, and the wooden board was arranged. The positions of the anemones, along the table as well as with respect to the lighted and shadowed halves, were then recorded at intervals, usually daily. The day after the wooden board was set in place was counted as the first day. The small number of animals occasionally found on the borderline between lighted and shadowed areas was divided equally between the two scores. The anemones were not fed.

Elimination of zooxanthellae

Maintenance in the dark, used successfully to eliminate symbiotic algae from various invertebrate hosts (see, *e.g.*, Zahl and McLaughlin, 1959), was not very satisfactory for *A. elegantissima* because of the long time required. The anemones were maintained in a darkroom in running sea water and exposed to dim light for only a few minutes every few weeks, in order to take sample bits of tissue, cut from the tentacles and sides of the column. Only after 12 weeks in the dark did most of the tissue samples clearly have fewer zooxanthellae than at the beginning; after nearly three times this long (34 weeks), a few zooxanthellae were still found. When the treatment was terminated at 48 weeks, no zooxanthellae were found in the tissue samples, nor did zooxanthellae return when these animals were subsequently kept in the light.

The most satisfactory method found for ridding the anemones of their symbiotic zooxanthellae was exposure to elevated temperatures. The anemones were kept in warm sea water for one to several days, then put back into running sea water at 13°–15° C for 2–3 weeks. During this time they egested mucus-wrapped pellets of zooxanthellae until no algae were found in their tissues. The anemones appeared healthy and normal throughout the treatment, as long as the heated sea water was aerated continuously and changed frequently. The effectiveness of exposure to temperatures from 25°–37° C, for varying periods of time, was tested (see Buchsbaum, 1968 for details). The time and temperature ranges that were both effective and well-tolerated by the anemones were very narrow; 30°–32° C for 48 hours was most successful. Such heat-treated anemones have been kept in the light for more than a year, never regaining any zooxanthellae, and this appears to be a convenient and reliable method of obtaining anemones without zooxanthellae for experimental purposes.

RESULTS

Sea anemones with zooxanthellae

Sea anemones with zooxanthellae, gathered from sunny intertidal habitats, always distributed themselves in the lighted half of the experimental area, whether the animals were initially equally distributed between the lighted and shadowed halves (Figure 1A) or placed entirely in the shadowed area (Figure 2A). The time course of the response varied in different experiments. The animals represented in Figure 1A were the slowest observed; 95% were found in the lighted area after 13 days. In another, similar experiment 93% were found in the lighted area after only 4 days. In the experiment represented in Figure 2A, although all

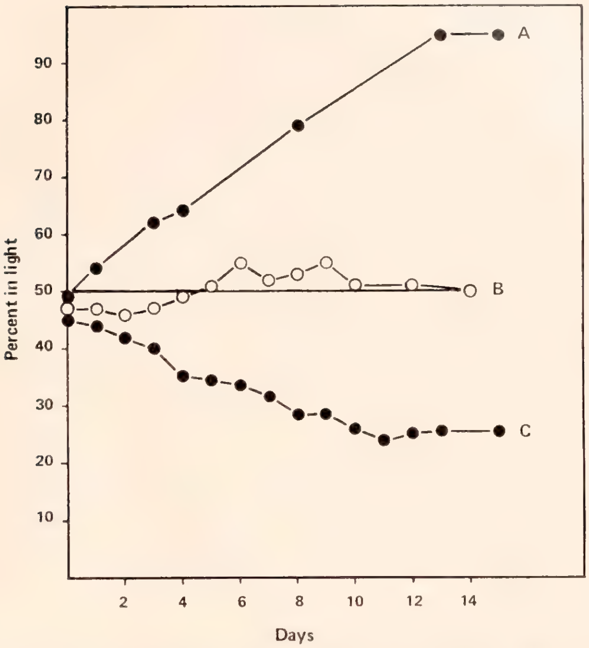

FIGURE 1. Percentage of anemones in lighted half of seawater table, after being initially randomly distributed. At 2 weeks: (A.) Anemones with zooxanthellae, from a sunny habitat, showed a significant positive light response ($\chi^2 = 30.4$, $P < 0.001$, N = 38). (B.) Anemones without zooxanthellae showed no significant light response ($\chi^2 = 0$, $P > 0.99$, N = 51); zooxanthellae were eliminated from these animals after exposure to elevated temperature. (C.) Anemones with zooxanthellae, from a shaded habitat (about 9% of light intensity measured in open intertidal), showed a significant negative light response ($\chi^2 = 16.0$, $P < 0.001$, N = 68).

of the animals were initially placed in the shadowed half, 92% had moved to the lighted area after only 5 days.

Sea anemones without zooxanthellae

Sea anemones without zooxanthellae distributed themselves randomly with respect to the lighted and shadowed areas. When equal numbers were initially placed in each half of the experimental area, their distribution remained random with respect to light (Figure 1B). When all were initially placed in the shadowed half, they slowly emerged into the lighted area until equally distributed between the lighted and shadowed portions (Figure 2B, C). Again, the time course varied in different experiments.

Sea anemones with few zooxanthellae

Sea anemones gathered from partially shaded intertidal habitats, with incident light down to about 10% of that measured in open intertidal areas, were found to harbor limited numbers of zooxanthellae. Such animals, when tested for photo-

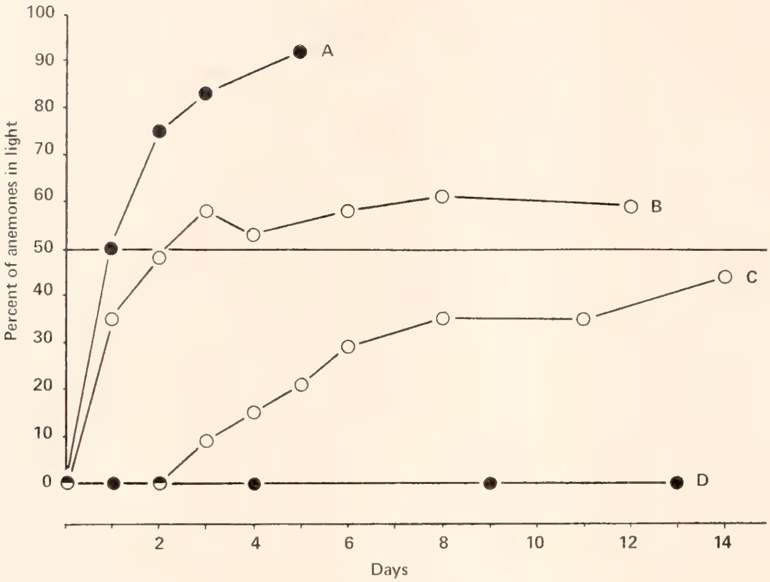

FIGURE 2. Percentage of anemones in lighted half of seawater table, after being initially distributed all in the shadowed half; (A–C) anemones with zooxanthellae when collected, from a sunny habitat, all from the same clone. (A.) Anemones with zooxanthellae showed a significant positive light response (at 5 days: $\chi^2 = 37.2$, $P < 0.001$, $N = 52$). (B.) Anemones having lost zooxanthellae after exposure to elevated temperature showed no significant light response (at 12 days: $\chi^2 = 1.2$, $P = 0.27$, $N = 41$). (C.) Anemones having lost zooxanthellae after maintenance in darkness showed no significant light response (at 14 days: $\chi^2 = 1.1$, $P = 0.30$, $N = 34$). (D.) Anemones with zooxanthellae, from a shaded habitat (about 9% of light intensity measured in open intertidal), showed a significant negative light response (at 13 days: $\chi^2 = 25.0$, $P < 0.001$, $N = 25$).

taxis as above, always occupied the shadowed half of the experimental area. If they were initially equally distributed between lighted and shadowed halves, they moved into the shadowed portion (Figure 1C). If initially placed entirely in the shadowed area, they remained there, none emerging into the light (Figure 2D).

Light intensity

The plasticity of behavior indicated by these results suggested the possibility that the anemones with zooxanthellae which I collected from shaded habitats, and which reacted negatively to the light intensity regularly used in the experiments (Figures 1C, 2D), might respond positively to a lower light intensity. To test this possibility, I selected anemones from a shaded habitat where midafternoon light readings were 200–250 foot-candles. Distributed randomly in a seawater table, these animals showed a negative response to the usual light intensity of 700 foot-candles, moving into the shadowed half of the table (Figure 3, left). After their distribution had remained stable for 2 weeks, the light intensity was reduced to 250 foot-candles; and within a few days the anemones began to move out into the lighted area, reversing from a negative to a positive response (Figure 3, right).

Elimination of zooxanthellae

All of the results just described suggested that phototaxis in these anemones depends directly on the presence of zooxanthellae. However, since naturally occurring anemones with and without zooxanthellae were collected from different habitats and belonged to different clones, several other hypotheses seemed possible. The response might depend on previous exposure to appropriate light intensities; pigments produced by the anemones only in the light might be involved (see Buchsbaum, 1968). The absence of phototaxis in animals from dark habitats might then reflect only their lack of previous exposure to light and not their lack of zooxanthellae. Alternatively, phototactic behavior might be developed only after the acquisition of zooxanthellae and then retained. In this case, animals without algae might display phototaxis if they had previously harbored zooxanthellae. Finally, phototaxis might have a genetic basis, since an individual that actively orients to light, either as a settling planula or attached adult anemone, is

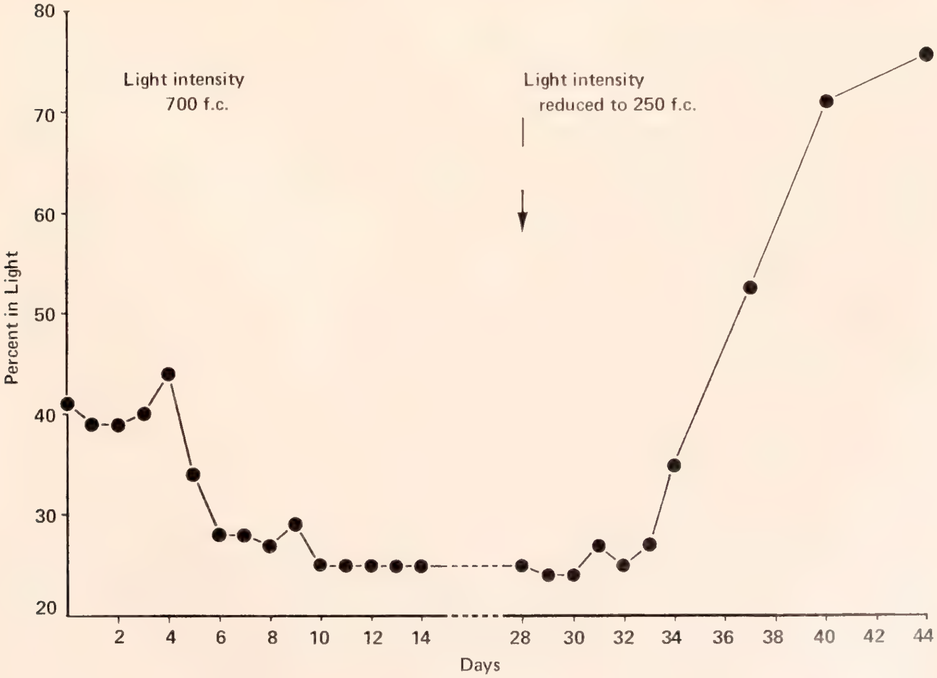

FIGURE 3. Percentage of anemones in lighted half of seawater table, after being initially randomly distributed. Anemones with zooxanthellae, from a shaded habitat (about 47% of light measured in open intertidal), showed a significant negative light response at 700 foot-candles (at 14 days: $\chi^2 = 12.2$, $P < 0.001$, $N = 51$) and later a significant positive light response at 250 foot-candles (after 16 days: $\chi^2 = 14.2$, $P < 0.001$, $N = 51$).

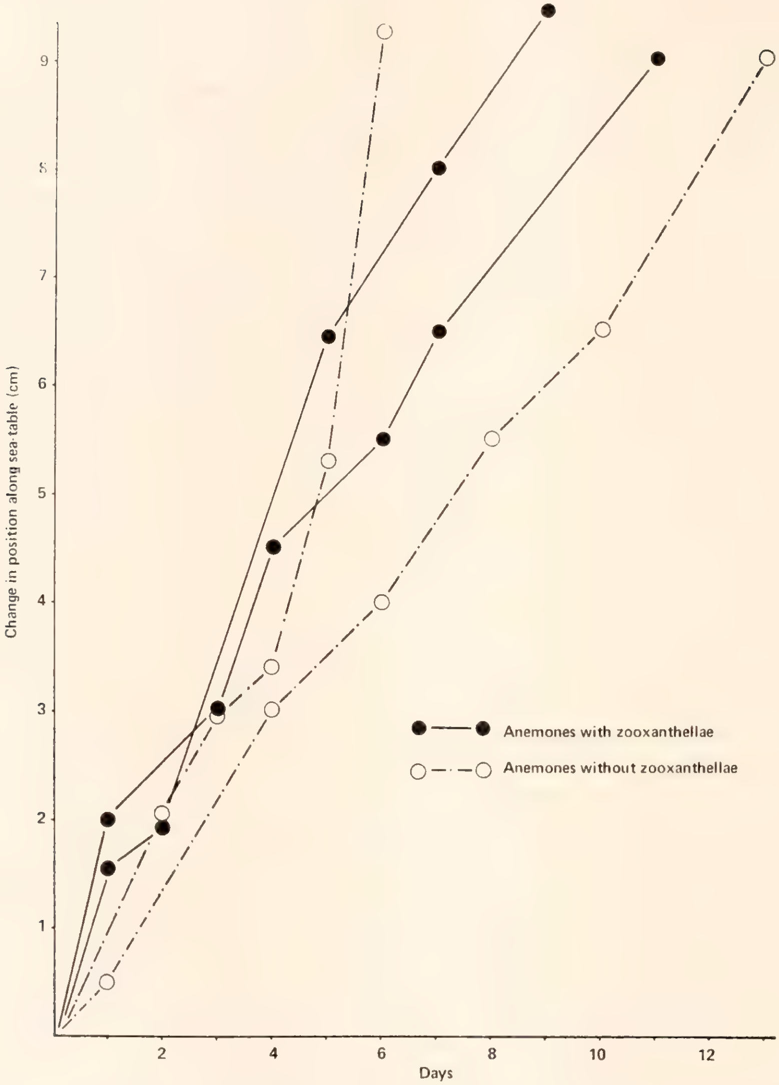

FIGURE 4. Movement of anemones up-current in the seawater tables. Data from 4 experiments with 4 different clones of animals (N = 34, 30, 38, 32). Changes in average position along the table (sum of anemone positions/no. of anemones) were taken as an index of movement, since individual anemones could not be conveniently followed.

more likely to become host to light-requiring symbionts than an animal that is indifferent to light.

To test these possibilities, I collected sea anemones from a single asexually produced clone, all with abundant zooxanthellae. Some were kept as controls in natural light in running sea water at ambient temperature. Others were induced to lose their zooxanthellae by subjecting them briefly to heat or darkness (see Methods). After these treatments, tissue samples examined by light microscopy revealed no zooxanthellae; nor did these anemones ever re-establish symbiosis with algae, although subsequently kept in the light.

All of the anemones—presumably genetically identical, collected from the same small intertidal area, and originally harboring many zooxanthellae—were tested for their response to light. The control individuals with zooxanthellae showed a positive phototactic response (Figure 2A), while those which had lost their zooxanthellae distributed themselves equally between lighted and shadowed areas (Figures 1B, 2B, C). I found no differences in phototactic behavior between anemones occurring naturally without zooxanthellae, collected from dark habitats, and anemones in which I induced loss of zooxanthellae by exposure to heat or darkness.

Activity controls

Another hypothesis considered was that the apparent failure of anemones without zooxanthellae to respond to light might simply reflect inactivity or slowness of movement. However, in experiments in which all animals were initially placed in the shadowed area, anemones with zooxanthellae moved into the light at a rate fast enough to reveal any light response within the duration of the experiment (Figure 2B, C) but with a pattern quite distinct from the positive and negative responses of the anemones with zooxanthellae (Figure 2A, D). Moreover, in all experiments, regardless of light-directed movements across the seawater table, the anemones moved along the table, against the current of sea water. This movement was followed by noting the position of each anemone along the table each time its position with respect to light was recorded. Changes in the positions of the anemones along the table in 4 experiments are represented in Figure 4. Although rates of movement varied in different experiments, there appeared to be no difference in the rates of anemones with and without zooxanthellae.

DISCUSSION

Under the conditions of these experiments, sea anemones with zooxanthellae always displayed phototaxis, either positive or negative depending on the light intensity. Anemones without zooxanthellae—even those that had previously harbored zooxanthellae and that were genetically identical clone-mates of phototactic individuals—never displayed phototaxis, appearing completely indifferent to light and shade. These observations indicate that phototactic behavior in the sea anemone *Anthopleura elegantissima* is not a fixed species character, but rather depends directly on the presence of endosymbiotic zooxanthellae.

The mechanism by which the symbionts influence their anemone hosts is unknown, but possibly involves some metabolite consumed or produced in photosynthesis by the algae. Production of oxygen or any organic compound, removal of carbon dioxide or other metabolic by-products, or release of any stimulant or inhibitor by the algae could all work in the same way. The difference in light intensity on two sides of the animal would result in different degrees of activity by the algae. The quantities of both animal and algal pigments vary with the light intensities under which the organisms live (Buchsbaum, 1968), and light intensities either higher or lower than the accustomed range might be expected to reduce algal photosynthesis. The gradient thus established within the animal would direct its movement. Determination of the action spectrum for phototaxis would provide evidence for or against some such photosynthetic mechanism. In the ciliate *Paramecium bursaria,* which responds phototactically only under conditions of limited oxygen, the evidence suggests a mechanism involving oxygen production by the symbiotic algae (Jennings, 1915; Stanier and Cohen-Bazire, 1957).

In this context it would be particularly interesting to know the phototactic behavior of specimens of *A. elegantissima* from the northwest coast of North America which are symbiotic with a green unicellular alga. Unlike zooxanthellae, which release approximately 50% of their photosynthetically-fixed carbon to the anemone tissues in a variety of organic compounds (Trench, 1971a, 1971b), these green symbionts fix carbon photosynthetically at appreciable rates but release less than 2% (Muscatine, 1971). Yet in symbiosis with both kinds of algae, the anemone tissues presumably receive oxygen and give up carbon dioxide and other inorganic nutrients to the algae. Tests for phototactic behavior in anemones with the green symbionts might therefore provide clues as to which algal activities influence phototaxis.

The symbiosis between *A. elegantissima* and zooxanthellae was the first in which transfer of photosynthetically-fixed carbon from an alga to its host was directly demonstrated (Muscatine and Hand, 1958). Studies since then have characterized the biochemistry of the algal contribution (Trench, 1971a, 1971b) and have shown that the zooxanthellae slow weight loss of the anemone during starvation in the light (Muscatine, 1961a; Buchsbaum, 1968). The physiological mechanism for a potential selective advantage in the symbiosis is thus well established. Phototaxis seems to be a behavioral mechanism ensuring the effectiveness and continued maintenance of the symbiosis.

If anemones with zooxanthellae are placed in the dark, the number of algal cells and total chlorophyll per unit weight of anemone tissue decrease, eventually to undetectable levels (Muscatine, 1961b; Buchsbaum, 1968). Intertidal anemones in a sunny habitat, if suddenly shaded (for example, by growing seaweeds or by debris), may avoid such reduction in symbionts by positive phototaxis. On the other hand, anemones with zooxanthellae adapted to partially shaded habitats, if suddenly exposed to increased light intensity, also suffer a reduction in numbers of algae and total chlorophyll, the tentacles and oral disk becoming especially pale. After a few days in full sun, microscopic examination of tentacle tissue reveals only rare, bleached algal cells (Buchsbaum, 1968). Shade-adapted anemones may avoid such loss of algae by negative phototaxis. How much these anemones actually move under normal conditions in the intertidal is not known, but their phototactic behavior in laboratory experiments suggests that they may effectively control their light environment in this way.

This study was supported by a predoctoral fellowship from the National Institutes of Health, carried out at the Hopkins Marine Station, Pacific Grove, California, and submitted to Stanford University as part of a doctoral dissertation. I am grateful to the faculty, staff, and students of the Marine Station, especially my advisory committee, Drs. J. H. Phillips, D. P. Abbott, and W. L. Lee; to Dr. J. S. Pearse for reading the manuscript; and to the Division of Natural Sciences, University of California, Santa Cruz for library and secretarial assistance.

SUMMARY

The sea anemone *Anthopleura elegantissima,* with and without endosymbiotic zooxanthellae, was tested for evidence of phototactic behavior. Anemones with zooxanthellae always displayed phototaxis, either positive or negative depending on the experimental light intensity and the light intensity of the habitat from which the animals were taken. Anemones without zooxanthellae—even those that had previously harbored zooxanthellae and that were genetically identical clonemates of phototactic individuals—never displayed phototaxis, appearing completely indifferent to light and shade.

The results indicate that phototaxis in this sea anemone depends directly on the presence of its symbiotic algae. It is suggested that the flexible phototactic behavior of the anemone may play an important role in favorably regulating the amount of light to which the zooxanthellae are exposed.

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MODIFICATION OF SEA ANEMONE BEHAVIOR BY SYMBIOTIC ZOOXANTHELLAE: EXPANSION AND CONTRACTION

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No specialized photoreceptors have been identified in sea anemones, but it has been demonstrated repeatedly that at least some anemones react to visible light (*e.g.*, Hargitt, 1907; Parker, 1917; Cotte, 1921, 1922; Batham and Pantin, 1950; North and Pantin, 1958; di Milia and Geppetti, 1964; Clark and Kimeldorf, 1970; Ottaway, 1973). And the most frequently reported reaction is contraction. Contraction is not a specific response to light, however. It is provoked by almost any strong stimulus that might be considered unfavorable: vigorous mechanical or electrical stimulation, a variety of chemicals, exposure to air, extremes of temperature, osmotic shock, X-radiation, etc. (*e.g.*, Torrey, 1904; Piéron, 1906; Fleure and Walton, 1907; Parker, 1917; Hall and Pantin, 1937; di Milia and Geppetti, 1964; Kimeldorf and Fortner, 1971).

A large number of anemones expand in twilight or darkness and contract in daylight. Parker (1917) reviews reports of a dozen genera, some with several species that have been observed to behave in this way; and 3 or 4 genera are reported to be indifferent to light. In contrast, I have found only a few reports of anemones that regularly expand by day. Bohn (1907, 1908a, 1910) affirms that specimens of *Actinia equina* from certain habitats expand in daylight and contract at night, while those from other habitats show exactly opposite behavior. Di Milia and Geppetti (1964) found that this species consistently expands in darkness and contracts in light. *Anemonia sulcata*, an anemone that harbors symbiotic zooxanthellae, is reported to expand in the light and contract in the dark (Gosse, 1860; Bohn, 1906; Smith, 1939). Finally, Gee (1913) observed regular expansion in light and contraction in darkness by specimens of *Anthopleura elegantissima* with zooxanthellae. There are similar reports for polyps of several gorgonians with zooxanthellae (Wainwright, 1967; Chapman and Theodor, 1969).

Various authors have propounded diverse views of the factors which influence expansion and contraction in sea anemones. The difficulties encountered may be illustrated by comparing Bohn's (1908b) listing of 36 environmental factors which affect expansion and contraction with Batham and Pantin's (1950) finding that expansion and contraction continue in the apparent absence of any environmental stimuli. I have made no attempt to resolve the hypothetical effects of all these factors. However, as part of a more extensive study on the symbiosis of *Anthopleura elegantissima* with zooxanthellae (Buchsbaum, 1968; Pearse, 1974), I have observed differences in the expansion of specimens with and without the endosymbiotic algae and have tried to evaluate the possibility that this behavior is related to the symbiosis.

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METHODS

Specimens of the sea anemone *Anthopleura elegantissima* (Brandt, 1835) with and without zooxanthellae were collected from rocky intertidal areas in central California, and zooxanthellae were eliminated from some specimens in the laboratory by exposure to elevated temperature, as detailed previously (Buchsbaum, 1968; Pearse, 1974).

Recording of expansion and contraction was simplified by the fact that the large majority of sea anemones were usually either fully expanded or fully contracted under experimental conditions. Arbitrary decisions were necessary only occasionally. The anemones were not fed.

Anemones were observed in the laboratory both in standing sea water and in running sea water. Light was provided by a bank of fluorescent tubes operated automatically on an alternating cycle (12 hours light: 12 hours dark). Observations during the dark period were by dim white or green (540 nm Klett filter) incandescent light; the period of exposure to these dim light sources was never more than a few seconds, and the anemones were not observed to react to them.

Standing sea water

Single animals were placed and allowed to attach in open glass jars 5 cm in diameter, containing about 100 ml sea water, in a temperature-controlled room at 12° C. The light intensity was about 450 foot-candles, as measured with a Photovolt 200 photometer, approximating midafternoon light readings of the intertidal habitat where the anemones with zooxanthellae were collected. Records of expansion and contraction of individual anemones were kept. Oxygen determinations by the Winkler method (Strickland and Parsons, 1965) were made using single animals in similar, sealed jars, under the same conditions of temperature and light (see Figure 1).

Running sea water

Groups of 30 animals each were placed and allowed to attach in open plastic boxes (20 × 28 cm, 10 cm deep), and the total number of animals expanded or contracted was recorded at intervals (see Table I, Figures 2 and 3). A continuous flow of sea water at 14° C ± 1° was maintained through the boxes. The light intensity was approximately 250 foot-candles, chosen as intermediate in the habitat range of the anemones in this experiment.

RESULTS AND OBSERVATIONS

Anemones in standing sea water

In observations over 8 days, the anemones without zooxanthellae, collected from a shaded intertidal habitat (midafternoon light intensity about 2 foot-candles), did not expand or contract in response to a daily cycle of light and darkness. At the beginning of the experiment, when supplied with fresh sea water, they stayed mostly expanded in light and dark. Left for the rest of the time in stagnant sea water, they contracted and remained so. In contrast, the anemones with zooxanthellae showed a very regular pattern of expansion in light and contraction in darkness throughout the experiment. The animals usually expanded within about 10 minutes after the light was turned on. Two anemones from which all zooxanthellae were eliminated in the laboratory were also observed; these animals behaved as did the anemones naturally occurring without zooxanthellae, remaining contracted throughout most of the experiment.

Anemones without zooxanthellae, even those that had been contracted for several days, expanded within minutes after the sea water was renewed or thoroughly stirred, or after air or oxygen was bubbled through. Bubbling nitrogen through the sea water did not stimulate expansion of contracted anemones.

The oxygen content of the sea water was measured in similar but sealed jars containing single anemones with and without zooxanthellae under the same conditions of light and temperature as in the behavior experiments. The oxygen data for experiments of 12 hours in light or dark (Figure 1) show that in the light, the oxygen content of the sea water increased substantially in jars containing sea anemones with zooxanthellae. Addition of light and dark values for each individual anemone with zooxanthellae gives mixed positive and negative results;

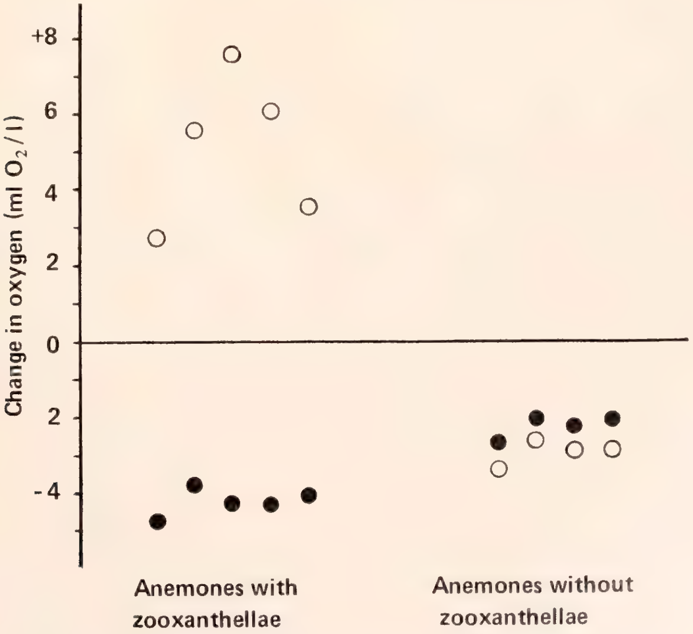

FIGURE 1. Changes in oxygen content of sea water surrounding 5 anemones with zooxanthellae and 4 anemones without zooxanthellae, in light (open circles) and darkness (solid circles), after 12 hours. The oxygen content of the sea water at the start of each experiment averaged 5.66 ml O_2/1 of sea water and decreased a maximum of 0.15 ml O_2/1 in control vessels containing no anemones. Values in the figure were corrected for changes in control vessels.

Table I

Expansion of anemones in light and dark in running sea water. Light intensities of the habitats from which the anemones were collected were measured under clear skies in midafternoon. The values for the percentage of anemones expanded are means and standard deviations of data for 10 days.

	Light intensity of habitat (foot-candles)	% Expanded	
		Light	Dark
Clone from a deeply shaded habitat			
Group 1, without zooxanthellae	2	100	95.7 ± 6.0
Clone from a habitat spanning a range of light intensities			
Group 2, without zooxanthellae	2	100	91.2 ± 7.6
Group 3, with few zooxanthellae	40	100	49.4 ± 13.0
Group 4, with zooxanthellae	210	99.6 ± 1.1	27.5 ± 9.9
Clone from an open habitat			
Group 5, with zooxanthellae	450	99.4 ± 1.5	23.8 ± 10.2
Group 6, without zooxanthellae (all eliminated in the laboratory)	450	98.9 ± 2.0	38.9 ± 11.8

but experiments run continuously for 24 hours (12 hours light + 12 hours dark) consistently yielded negative oxygen values, suggesting microbial growth.

Anemones in running sea water

Expansion and contraction data from observations of anemones in running sea water are presented in Table I. Virtually all of the anemones, both with and without zooxanthellae, expanded in the light. Anemones without zooxanthellae, collected from shaded intertidal habitats (Groups 1 and 2), remained expanded in the dark also. In contrast, the large majority of anemones with abundant zooxanthellae (Groups 4 and 5) contracted in the dark, just as they did in standing sea water experiments. The anemones from an intermediate habitat with dim light, containing few zooxanthellae (Group 3), showed intermediate behavior; in the dark, about half remained expanded and half contracted.

The anemones that had previously harbored zooxanthellae, but had been caused to eliminate the algae in the laboratory (Group 6), did not remain continuously expanded as did the anemones naturally occurring without zooxanthellae. Rather, like the anemones with zooxanthellae, the majority of anemones that had lost their zooxanthellae expanded in the light and contracted in the dark.

The time course of expansion and contraction is illustrated in Figures 2 and 3. A majority of the anemones responded within 10 minutes after the lights were turned on or off, and by 20–30 minutes, all had usually responded. However, during the 12 hours of continuous light or darkness that followed, some variability in behavior often appeared, especially in the dark. This variability is evident in the observations summarized in Table I, which were always made toward the middle or end of the light and dark periods. This procedure was chosen in order to record a sustained level of response rather than the more dramatic 0% or 100% expansion that was usually observed immediately after a change in light phase. If the lights were turned on or off in the middle of a 12-hour dark or light period, both the time course and percentage of anemones responding were the same as when the lights went on or off after the usual 12-hour interval.

Field observations

Field observations were limited to anemones with zooxanthellae found in tidepools at low water, since animals exposed to air by the receding tide almost invariably contract, and no anemones without zooxanthellae were found in suitable pools. In pools viewed at dawn, all of the anemones were contracted. As the daylight gradually increased, they suddenly began to expand, until all were fully expanded after 20–30 minutes. The time course of dawn expansion in the field under a gradually increasing light intensity was thus the same as the time course in the laboratory, where the lights were turned on suddenly (Figure 2). On slightly overcast days, the anemones remained fully expanded. However, on clear days, the animals contracted in midday sunlight. At such times, the distribution of expanded and contracted anemones in the pools corresponded exactly to the pattern of shadow and sunlight, respectively. No temperature differences in the sea

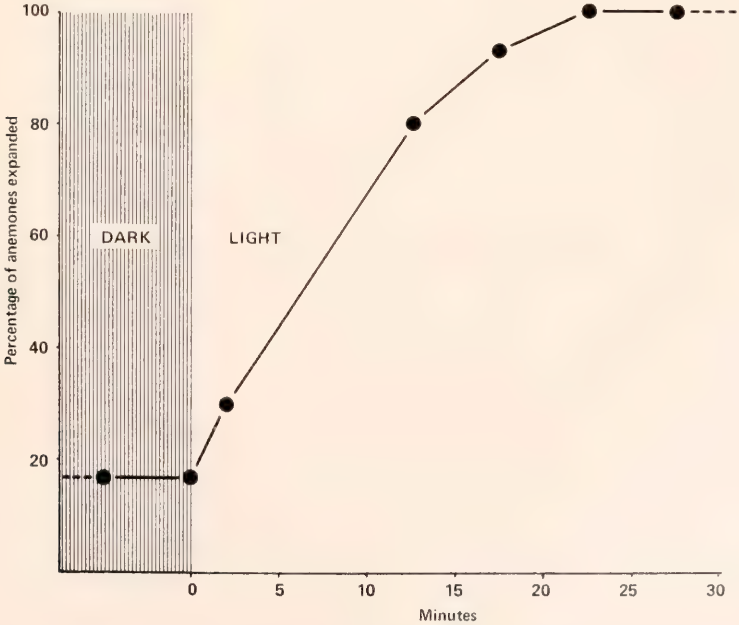

FIGURE 2. Time course of expansion by sea anemones in running sea water. The data presented are from a group of anemones with zooxanthellae, but the time course was the same for anemones that had lost their zooxanthellae.

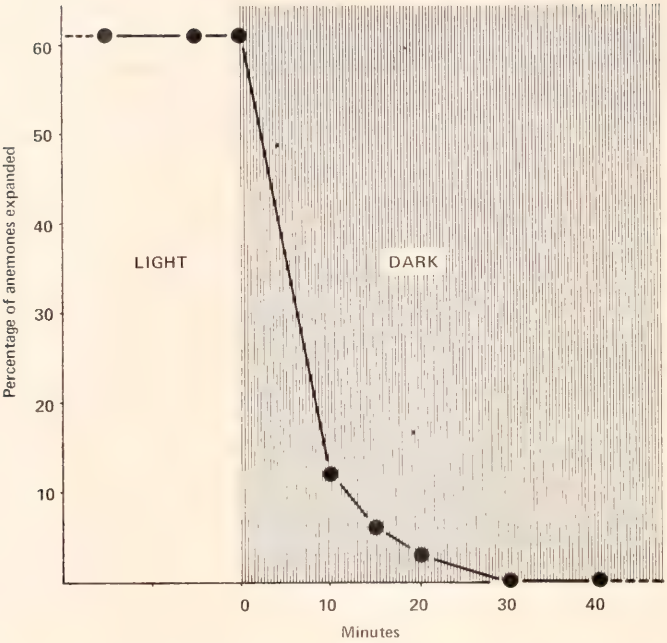

FIGURE 3. Time course of contraction by sea anemones in running sea water. The data presented are from a group of anemones with zooxanthellae, but the time course was the same for anemones that had lost their zooxanthellae.

water could be found to account for the distribution; however, internal temperatures of the anemones were not determined.

Observations on anemones in outdoor tables and tanks with running sea water supplemented intertidal observations. Anemones with zooxanthellae expanded in diffuse daylight but contracted in direct sunlight; they contracted on moonless or cloudy nights, but expanded fully in bright moonlight. Anemones from which zooxanthellae had been eliminated followed the same pattern. The behavior of anemones without zooxanthellae originating from dark habitats was erratic.

DISCUSSION

Observations on the sea anemone *Anthopleura elegantissima,* in both standing and running sea water, have shown differences in the behavior of individuals with and without zooxanthellae. Anemones without zooxanthellae, originating from dark habitats do not regularly expand or contract with changes in light. In contrast, anemones with zooxanthellae expand in moderate light and contract in intense light or darkness, with striking uniformity. I have found also that individuals without zooxanthellae do not display phototaxis, while those with zooxanthellae show positive or negative phototaxis depending on the light intensity

(Pearse, 1974). The pattern of expansion and contraction in this sea anemone is thus similar to that of its phototactic behavior in that both are modified by symbiosis with zooxanthellae. In both kinds of behavior, anemones without zooxanthellae appear basically indifferent to light while anemones with zooxanthellae engage in active, light-related responses.

Behavior in both standing and running sea water suggests that the anemones without zooxanthellae generally expand in well-oxygenated sea water and contract when oxygen levels fall. A direct response to oxygen is further implicated by the rapid expansion of contracted individuals in stagnant sea water after renewal or stirring of the sea water or gassing with air or oxygen, and by the lack of expansion after gassing with nitrogen, which provided similar mechanical stimulation.

Anemones with zooxanthellae may also be responding to changes in oxygen concentration, rather than directly to light. The oxygen data in Figure 1 indicate that in the light photosynthesis by zooxanthellae met the respiratory requirements of both algae and anemone and raised the oxygen content of the sea water, as has been found in other cnidarians with zooxanthellae (*e.g.*, Yonge, Yonge, and Nicholls, 1932; Smith, 1939; Kanwisher and Wainwright, 1967; Roffman, 1968; Chapman and Theodor, 1969). In the dark, with the zooxanthellae no longer producing oxygen, its concentration fell. However, most of the anemones were contracted after only 10 minutes of darkness (see Figure 3), and it seems unlikely that oxygen content was much reduced in that short time. In running sea water, where anemones without zooxanthellae hardly ever contracted, it is especially difficult to explain contraction of anemones with zooxanthellae by a hypothesis of oxygen deprivation, unless they require much more oxygen. However, they may respond simply to a sudden reduction of available oxygen, regardless of absolute concentration. In the dark, the lower oxygen values recorded for anemones with zooxanthellae (Figure 1) probably reflect only the larger size of these particular individuals, compared with those lacking zooxanthellae; the respiratory burden added by the zooxanthellae was not determined. Use of specific photosynthetic inhibitors (see, *e.g.*, Vandermeulen, Davis, and Muscatine, 1972) or determination of the action spectrum of the response (see, *e.g.*, Clark and Kimeldorf, 1970) may help to establish whether expansion and contraction are direct responses to light or to some consequence of photosynthesis.

In standing sea water, accumulation of metabolic by-products excreted by the anemones might also lead to contraction. There is evidence that other cnidarians excrete less phosphorus (Yonge and Nicholls, 1931a, 1931b; Smith, 1939; Yamazato, 1966) and less nitrogen (Kawaguti, 1953; Muscatine, in press) when zooxanthellae are present, and that at least some respiratory carbon dioxide is fixed by zooxanthellae (Pearse, 1970). In the light, individuals of *A. elegantissima* with zooxanthellae excrete into the sea water only about half as much ammonia as do those lacking zooxanthellae, nitrogen being recycled between host and algal cells (L. Muscatine and C. D'Elia, University of California, Los Angeles, personal communication), and it seems likely that excretion of phosphorus compounds and carbon dioxide is also reduced. Accumulation of excretory products might also influence contraction indirectly through enhancement of microbial growth in the jars of standing sea water. Comparison of oxygen data from 12- and 24-hour experiments suggests that microbial respiration was considerable; and I observed that the sea water in the jars containing anemones without zooxanthellae became cloudy after several days, while that in the jars containing anemones with zooxanthellae remained clear.

However, if a direct response to change in available oxygen or any other consequence of photosynthesis by zooxanthellae were the only factor regulating expansion and contraction, the anemones from which zooxanthellae had been eliminated would be expected to behave exactly like anemones naturally occurring without zooxanthellae. This was indeed observed in standing sea water, where oxygen depletion or accumulation of excretory products could rapidly have become a determining factor. But in running sea water, like anemones with zooxanthellae, individuals that had lost their zooxanthellae expanded in light and contracted in darkness. This suggests the possibility that the anemones' behavior was conditioned by their previous symbiosis. Also suggestive of conditioning were the apparent changes in the behavior of anemones naturally occurring without zooxanthellae, which took place after prolonged exposure, not to endosymbiotic algae, but to seaweeds in intertidal pools and diatoms in laboratory vessels (see Buchsbaum, 1968 for details). I did not carry out any experiments specifically designed to test for evidence of conditioned behavior in these animals. However, in view of the number of recent suggestions that cnidarians are capable of simple forms of learning (see review by Rushforth, 1973), the expansion and contraction of these sea anemones seems a promising experimental system to investigate further.

How contracting in the dark might be of special selective advantage to a sea anemone with zooxanthellae is obscure. Decreased oxygen consumption has been reported in contracted sea anemones (Piéron, 1908; Shoup, 1932; Smith, 1939), including *Anthopleura elegantissima* (see Buchsbaum, 1968 for details). An intertidal anemone such as *A. elegantissima*, which often produces dense populations in isolated tidepools, may be subjected to low oxygen conditions during low tides, especially at night. Since oxygen consumption by at least some anemones (Sassaman and Mangum, 1972), including *A. elegantissima* (J. J. Childress, University of California, Santa Barbara, personal communication), is proportional to the oxygen concentration of the surrounding sea water, it might be to the anemone's advantage to reduce its rate of oxygen consumption by contracting when the oxygen concentration first begins to drop. However, these anemones appear to survive low oxygen conditions for long periods, and contraction may simply conserve energy.

Possible selective advantages of the anemones' behavior in light are easier to defend. Under moderate light intensities, expansion exposes the abundant zooxanthellae in the tentacles and oral disk to maximum illumination and thus presumably favors maximum photosynthesis. Oxygen data from gorgonians suggest that less light reaches the zooxanthellae in retracted polyps than in extended ones, due to shading by ectoderm and spicules, especially in strongly pigmented species (Kanwisher and Wainwright, 1967; Chapman and Theodor, 1969). In intense light, however, maximum exposure of zooxanthellae may instead reduce photosynthesis. Roffman (1968) suggests that in scleractinian corals with zooxanthellae, midday decreases in photosynthesis are related to extended exposure to high light intensity. Thus, contraction by *A. elegantissima* in bright sunlight may serve a significant function in shielding the zooxanthellae in the endoderm beneath the heavily green-pigmented ectoderm of the column of the animal. Ectodermal pigments are produced by these anemones under the influence of bright light, and poorly pigmented individuals from partially shaded habitats suffer substantial reduction in number of zooxanthellae and total chlorophyll when suddenly exposed to increased light intensities (Buchsbaum, 1968). The sea anemone *Actiniogeton sesere*, which occurs in shallow waters of Hawaiian reef flats and harbors zooxanthellae, produces extremely heavy concentrations of a similar green pigment in the tentacles and oral disk; these anemones remain expanded in full sunlight (Buchsbaum, 1968).

Development of pigmentation (Buchsbaum, 1968) and phototaxis (Pearse, 1974) may represent two mechanisms by which the sea anemone *Anthopleura elegantissima* favorably regulates the quantity of light to which its symbiotic zooxanthellae are exposed. These are both relatively slow responses and probably serve principally to permit the animal to select and adapt to habitats in a wide range of light intensities. Expansion and contraction provide a more rapid and flexible means for regulating the light reaching the zooxanthellae as light levels rise and fall from minute to minute throughout the day.

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Summary

The pattern of expansion and contraction by the sea anemone *Anthopleura elegantissima* differs in individuals with or without endosymbiotic zooxanthellae. Anemones without zooxanthellae, found in dark habitats, do not regularly expand or contract under changes in light. Anemones with zooxanthellae expand in moderate light and contract in intense light or in darkness, with striking uniformity.

However, this behavior does not always depend directly on the presence of zooxanthellae. Anemones that have previously had endosymbiotic zooxanthellae subsequently expand and contract with changes in light in the absence of these algae. Thus, conditioned responses may be involved.

It is suggested that expansion and contraction of the anemones may play an important role in favorably regulating the amount of light to which their zooxanthellae are exposed.

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SOME CONSEQUENCES OF SEXUAL DIMORPHISM: FEEDING IN MALE AND FEMALE FIDDLER CRABS, *UCA PUGNAX* (SMITH)

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The males of *Uca pugnax* (Smith), a fiddler crab species found along the coast of the northwest Atlantic Ocean, have one large chela used for mating displays, sound production, and aggression (Altevogt, 1955; Crane, 1941, 1943, 1967; Pearse, 1912; Salmon, 1967), and one small feeding chela. The female fiddler crab has two small feeding chelae. The crabs feed by picking up bits of mud and detritus from their habitat using the feeding chelae (Miller, 1961).

Kingsley (1888), Hyman (1920), Swartz and Safir (1915), Pearse (1912), Altevogt (1955) and Ono (1968) observed that females show more feeding motions than males, presumably because both feeding claws can operate alternately with almost continuous delivery of food particles to the mouth. If metabolic demands of both sexes are similar, the sexual dimorphism in the feeding claws must have forced the males to evolve some compensatory mechanisms such that food assimilation equals that of females.

Male fiddler crabs could increase food ingestion by having faster feeding motions, by feeding for a longer period of time than females, by feeding on larger mouthfuls or by having a higher assimilation efficiency of the food they do ingest. This paper first attempts to determine if there are any differences in metabolic rates, food intake, fecal production or digestive efficiency between male and female *U. pugnax* and then ascertains whether differences in duration of feeding or morphological dimorphisms involved in feeding exist between the two sexes.

METHODS

All crabs used in this study were collected at Great Sippewissett marsh in Falmouth, Massachusetts, in early fall, were brought to the laboratory and kept in seawater tables at room temperature. Activity cycles in fiddlers may last as long as one week away from water (Bennett, Shriner and Brown, 1967). All experiments were conducted at times corresponding to low tide, the time of maximum activity in the field.

Respiratory rates were measured with an oxygen electrode and chart recorder connected to a respiration chamber consisting of a 250 ml plastic cylinder stoppered at both ends. Demeusy (1957), Teal (1959), Vernberg (1959), Vernberg and

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Vernberg (1966) and Teal and Carey (1967) have previously measured respiration in *Uca.* Crabs in the present study were grouped by sex and size. Two crabs of the same sex and differing by no more than 0.25 mm in carapace width were placed in the seawater-filled chamber. A perforated stopper was pressed down into the chamber until all bubbles were excluded. The electrode was then inserted through the perforation. The water was stirred to avoid saturation of the electrode (Kanwisher, 1962). Temperature was measured before and after the 15 min. period during which the crabs were held in the chamber for respiration measurements.

Dry weight of males and females were obtained. To ensure reasonable comparisons the weight of the sclerotized parts of one of the chelae was subtracted from total weight. This eliminated the bias due to the enlarged male claw. The sclerotized portion of the chelae was measured by carefully removing muscle tissues and weighing the dried hard parts.

Size-matched crabs were starved two days and placed in individual fingerbowls to measure the amount of sediment ingested by male and female crabs of similar size. Three grams of marsh mud resuspended in water were placed with the crabs in 1 cm of seawater. The crabs were left undisturbed for six hours after which they were removed, along with any fecal pellets deposited. The remaining mud was redried and weighed.

The number of fecal pellets produced by crabs per unit time and the amount of organic matter in the pellets were measured to provide some idea of the amount of food processed and the efficiency with which organic matter contained in ingested mud was assimilated by males and females. About 20 g of wet marsh mud were placed in a fingerbowl containing seawater and offered to 3 male and female crabs of three size classes. The crabs were allowed to feed for five days under a regime of 12 hrs of dark and 12 hrs light. Fecal pellets were collected every three hours. The volumes of the fecal pellets as well as their dry weight were obtained. Whole fecal pellets were then combusted in a muffle furnace at 500° C for 24 hrs to allow the calculation of percent organic matter. The organic content of the mud on which the crabs fed was also measured.

Further measurements of the number of pellets were obtained by cutting 4 cm deep, 20 cm diameter cores of marsh turf, placing them in fingerbowls, and allowing crabs to feed on the surface sediments. Field measurements of pellet production were carried out by first locating single-entrance burrows by pumping seawater through the entrance and observing if water flowed out of any neighboring burrows. Once single entrance burrows were found, 6 inch aluminum flashing was used to construct a circular enclosure 20 cm in diameter. The marsh vegetation was removed from the enclosure and all fecal pellets were removed from the sediment prior to starting. The number of pellets was counted at feasible intervals over a 24 hr period.

The number of feeding motions of male and female crabs was recorded by observing individuals feeding on marsh sediment on a shallow seawater table for two minutes. Wooden barriers with narrow viewing slots were erected around the tables to prevent disturbing the crabs. Feeding motions consisted of a single chela moving from the sediment to the buccal cavity.

Table I

Regression lines fitted to values of partial pressure of oxygen (mm Hg) in the respiration chambers during a 15 min period for male and female crabs of various size classes. Initial temperature was 20° C, final temperature 21° C. Mean weights were calculated as dry weight minus 88.5% of the larger claw.

Carapace width class (mm)	Male crabs	Female crabs
13.3–13.5	16.0–0.08 × 0.25	16.3–0.08 × 0.26
13.8–14.0	15.9–0.08 × 0.27	15.7–0.10 × 0.31
14.0–14.3	16.0–0.11 × 0.30	16.1–0.11 × 0.31
14.5–14.8	15.6–0.15 × 0.32	15.6–0.12 × 0.34

The duration of feeding was measured by recording feeding activity of crabs placed in three shallow seawater tables. Each table was observed for 15 min repeating the sequence of observations five times until 75 min of observation per table were completed. Each observation period was divided into fifteen 1 min. segments. If a crab moved its feeding claws from the mud to its buccal cavity during any 1 min interval, a feeding unit was recorded. The feeding units for each crab were then summed for the five observation periods.

To obtain an indirect measure of food-gathering ability the length of the feeding chelae and the width of the flat ventral tip of the dactyl of the feeding claw were measured. The carapace width of each specimen was recorded as well.

RESULTS

Metabolic rates and food processing

There were no significant differences in the rates at which male and female crabs of similar size depleted oxygen (*t* tests on the slopes of the regression lines, $P > 0.05$, Table I). The very slight differences in weight between males and females were not significant.

The amount of mud ingested by starved male and female crabs did not differ significantly when tested with a paired *t* test (Table II).

The number of fecal pellets produced by male and female crabs (Table III) was variable but a two-way analysis of variance of the data from the laboratory

Table II

Amount of sediment ingested by starved U. pugnax *during a six-hour feeding period in the laboratory.*

Carapace width (mm)	g of sediment ingested Female crabs	 Male crabs
18.1	0.47	0.35
12.6	0.50	0.47
22.7	0.19	0.37
$\bar{x} \pm$ s.e.:	0.39 ± 0.10	0.40 ± 0.04

TABLE III

Number of fecal pellets produced by individual crabs per 24 hrs, feeding on loose mud obtained from the surface of Great Sippewissett marsh, in the surface of a core of marsh sediments taken into the laboratory and within field enclosures. The values in parenthesis are the number of individual crabs involved.

	Size of crabs expressed as carapace width (cm)	Mean No. of fecal pellets/24 hrs ± st. error	
		Female crabs	Male crabs
Crabs feeding on loose detritus in lab:	0.8–0.9	172 ± 58 (2)	119 ± 11 (2)
	1.2–1.3	53 ± 6 (2)	123 ± 31 (2)
	1.6–1.7	47 ± 13 (2)	98 ± 33 (2)
Crabs feeding on surface of marsh core in the lab:	1.2	129.5 ± 2.5 (1)	155.5 ± 23.5 (1)
	1.3		174.5 ± 4.5 (1)
	1.4		117.6 ± 3.5 (1)
Crabs feeding on marsh surface within field enclosures:	0.6	176 (1)	
	1.2–1.3	143.5 ± 37.5 (2)	157.2 ± 15.2 (4)
	1.6		177.5 ± 3.5 (2)
	1.9–2.1	142 (1)	110.8 ± 11.0 (4)

crabs fed on loose detritus showed that neither sex nor size of crabs significantly affected the number of fecal pellets obtained within 24 hrs. No differences are seen in the values obtained for the field measurments or the laboratory crabs feeding on the surface of a marsh core (Table III).

The volume and weight of fecal pellets (Table IV) were affected significantly by size of crabs ($F = 29.9$ and 4.15 respectively, both with 2 and 6 d.f.) but not by sex ($F = 0.8$ and 4.5 respectively, with 1 and 6 d.f.). The amount of organic matter (Table IV) in the pellets is not significantly related to either size or sex of crabs. There was no significant interaction between sex and size of fiddler crabs in any of these experiments.

These results suggest that male and female fiddlers did not have different metabolic demands and that the amount of bulk food processed by each sex is

TABLE IV

Volumes, weights and percent organic matter of fecal pellets for fiddler crabs. Six to 15 pellets were used from each of two crabs in each treatment combination. Tabled values are mean ± standard errors.

Size of crabs expressed as carapace width	Female crabs			Male crabs		
	Vol/pellet (10^{-5} cm^3)	Wt/pellet (10^{-5} g)	% organic matter	Vol/pellet (10^{-5} cm^3)	Wt/pellet (10^{-5} g)	% organic matter
0.8–0.9 mm	56 ± 15	33.1 ± 3.1	31 ± 0.2	83 ± 15	46.3 ± 2.8	34.6 ± 1.1
1.2–1.3 mm	172 ± 22	77.3 ± 23.3	34.5 ± 0.9	222.5 ± 30.5	78.9 ± 7.4	29.2 ± 4.1
1.6–1.7 mm	364.5 ± 40.5	112.8 ± 1.2	35.1 ± 0.7	349.5 ± 33.5	150.8 ± 1.8	32.8 ± 0.1

TABLE V

Number of feeding motions per 2 min observation period. Tabled values are mean plus or minus standard errors.

Carapace width	No. of individuals observed	No. feeding motions per 2 min	
		Females	Males
13–14 mm	16 males, 16 females	64.6 ± 2.0	33.0 ± 1.1
14–15 mm	15 males, 15 females	68.9 ± 2.2	38.5 ± 1.2

similar. Further, neither sex was more efficient at removing organic matter out of the ingested sediment. The organic content of the sediment on which the crabs fed was 23.7%, considerably lower than that of the pellets. This probably indicates selective feeding on particles of high organic content. There is a possibility that male crabs are more selective than females, choosing only the richest particles, but we have no evidence for this.

Feeding behavior

Table V shows that female *U. pugnax* carry out almost twice as many feeding motions as males during comparable spans of time. This was due to almost simultaneous feeding with two feeding appendages in the female. Males did not therefore use faster feeding motions to compensate for using only one feeding claw. Although there was considerable variation among individual crab, males did feed roughly twice as long as females, since male crabs scored about twice the feeding units awarded to females (Table VI).

Feeding morphology

The length of the feeding chela of males and females do not differ for specimens of comparable carapace width (Fig. 1). However, the width of the small flat ventral area on the tip of the dactyl of feeding claws is larger in males than in females (Fig. 2). The slopes of the regression lines are similar but the values for male *U. pugnax* lie above those for females for any comparable carapace width. This

TABLE VI

Number of feeding units recorded for a period of 75 minutes and three different seawater tables. Six male and female crabs were used in each table. See text for scoring of feeding units.

Table no.	Female crabs		Male crabs		Ratio male/female feeding units
	Range of carapace widths (mm)	No. of feeding units	Range of carapace widths (mm)	No. of feeding units	
1	15.1–17.4	6.8 ± 2.7	15.7–20.9	13.0 ± 2.7	1.9
2	14.2–18.3	7.7 ± 2.3	14.8–17.2	16.8 ± 4.6	2.2
3	14.0–17.1	3.7 ± 0.6	14.7–19.5	7.8 ± 1.1	2.1

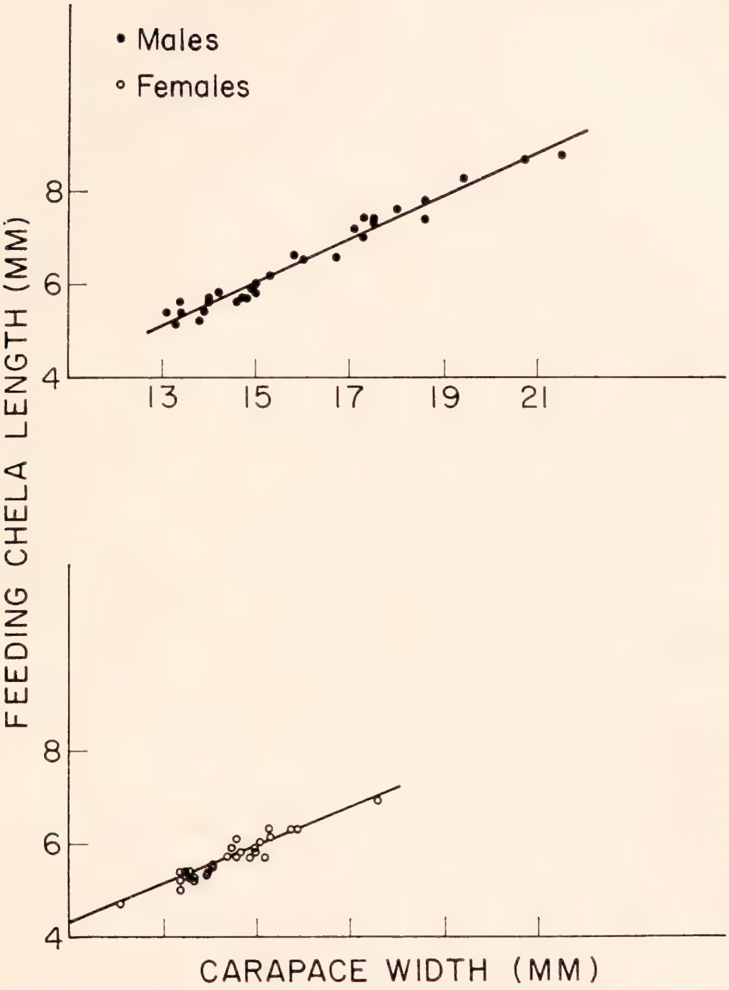

FIGURE 1. Length of feeding claws versus carapace width in male and female *Uca pugnax*. The lines show the calculated regression line for the points.

might make it possible for males to obtain larger mouthfuls per feeding motion than females.

DISCUSSION

Our results suggest that male and female fiddler crabs have similar nutritional demands, food-processing abilities, and digestive efficiencies. Since males have only one functional feeding claw, the number of feeding motions is half that observed in female *U. pugnax*. Males seem to compensate by extending the duration of feeding behavior. In the field feeding occurs mainly at low tide during daylight

(Ono, 1968, and our own observations). We have observed males feeding underwater in Massachusetts and Teal (1958) saw crabs feeding during high tides in Georgia. In the marshes of Buzzards Bay along the coast of Massachusetts, mud flats and grass-covered habitats in which *Uca* feed are exposed for about four hours at low tide. We have observed that as high tide approaches only males are still feeding. This agrees well with our results of prolonged feeding with males.

The two-fold increase in feeding duration would seem to be enough compensation for lack of a feeding appendage. However, the morphology of the feeding claw is such that males may be able to grasp and feed on larger mouthfuls per feeding motion.

Longer feeding periods may expose males to greater predatory mortality. However, in a sample of 713 specimens of *U. pugnax* from Great Sippewisset Marsh there were more males than females present (42.9% females). This sex ratio was very similar to that found by Shanholzer (1973) in a salt marsh in Georgia and is not unusual among marine invertebrates (Wenner, 1972). Data now in preparation from Great Sippewissett show greater mortality of the smaller females than males. The need to repeatedly expose eggs to the flow of well-oxygenated water may increase the exposure of berried females to predatory mortality by forcing females to remain in the relatively unprotected creek banks at high tide. The egg masses also may impede locomotion of females and increase

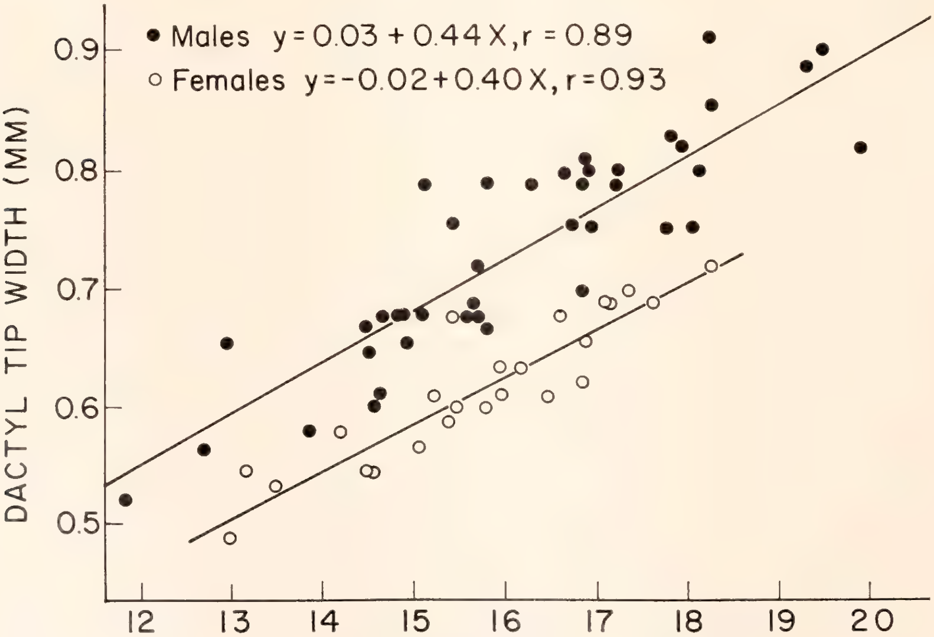

FIGURE 2. Width of the tip of the feeding dactyl versus carapace width in male and female *Uca pugnax*. The lines show the calculated regression line for the points.

their vulnerability to predators (Hyman, 1920). It may be, however, that differential mortality between the sexes does not affect the sex ratios (Leigh, 1970) so that sexual dimorphism does not directly lead to different survivorship rates.

There are no doubt seasonal fluctuations on the metabolic expenditures of male and female crabs related to sexual dimorphism. Males show great activity during breeding displays (Crane, 1958, 1966; Salmon, 1965), while berried females not only produce eggs from late May to early July but must, as already mentioned, care for the egg masses. To fully develop the consequences of sexual dimorphism these changes over time must be considered. This paper merely illustrates that at any one time there are mechanisms operating by which males compensate for the lack of one feeding appendage, and the results also evidence the complex consequences of apparently simple morphological and behavioral differences.

Summary

1. There were no differences in the respiratory rates of male and female *Uca pugnax* of comparable sizes.

2. The amount of salt marsh sediment ingested by starved male and female crabs was similar.

3. The number and weight of fecal pellets produced by male and female crabs were similar, as was the organic matter content.

4. The above suggests that there are minimal differences in food demands and digestive efficiencies between the sexes, yet the enlarged claw of the fiddler crabs cannot be used for feeding. This requires some compensatory mechanism in male crabs.

5. Male fiddlers do show about half the feeding motions per unit time compared to females, but they compensate by feeding about twice as long. This is corroborated by field observations.

6. Further compensation, if needed, could be achieved by the slightly larger holding surface of the feeding claw in males, perhaps allowing the grasping of larger fragments of marsh sediment.

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STUDIES ON THE REPRODUCTIVE SYSTEMS OF SEA-STARS. I. THE MORPHOLOGY AND HISTOLOGY OF THE GONAD OF *ASTERIAS VULGARIS*[1]

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Although the gonads of *Asterias vulgaris* are part of one of the major organ systems in this sea-star they have not been the subject of a basic morphological or histological study other than the preliminary account published by Field in 1892. The structure of the reproductive system has been described with varying degrees of completeness in a wide variety of other sea-stars (*Asterias rubens,* Hoffmann, 1872; *Asterias rubens* and *Asterina gibbosa,* Ludwig, 1877, 1878; *Asterias rubens,* Hamann, 1885; *Asterina gibbosa,* Cuénot, 1887; *Solaster endeca* and *Asterias rubens,* Gemmill, 1911, 1912 and 1914; *Asterina batheri,* Hayashi, 1935; *Coscinasterias tenuispina,* Cognetti and Delavault, 1958; *Echinaster sepositus,* Delavault, 1960; *Marthasterias glacialis,* Delavault and Cognetti, 1961; *Odontaster validus,* Pearse, 1965; *Pisaster ochraceus,* Mauzey, 1966; *Asterina gibbosa* and *Echinaster sepositus,* Tangapregassom and Delavault, 1967; *Leptasterias hexactis,* Chia, 1968; *Asterina gibbosa,* Bruslé, 1969; *Henricia sanguinolenta,* Chia, 1970; and *Astropecten armatus, Henricia leviuscula, Patiria miniata, Pisaster ochraceus,* Davis, 1971), but no comprehensive, detailed, and accurate description has been published for any sea-star which discusses the structure of the gonads based on observations with both the light and electron microscopes.

In the present study, observations have been made on both sexes of *Asterias vulgaris,* involving principally the morphology and histology of the gonads; notes are included on the ultrastructure of the tissues of the gonad and also on modifications occurring in the structure of the gonad during a single annual reproductive cycle. The information provided here should be useful to students of the physiology of reproduction in asteroids and will also provide a basis for comparative studies on the reproductive system in other asteroids.

MATERIALS AND METHODS

Collection and identification of specimens

All specimens of *Asterias vulgaris* used in this study were collected at the Isles of Shoals, a group of islands in the Gulf of Maine about 10 miles southeast of Portsmouth, New Hampshire. The principal collection site for this work was the leeward face of a breakwater stretching in a north-south direction from Cedar Island to Star Island. A series of four collections of sea-stars (referred to as "major collections") was made at this site in October and December, 1970 and in

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[1] This paper represents part of a thesis submitted to the Graduate School of Cornell University in partial fulfillment of the requirements for the degree of Master of Science.

March and July, 1971. Information gathered by Sherman (1966) about the reproductive cycle of *Asterias vulgaris* on the north side of Cape Cod was used as a guide in selecting the dates of these collections. At her study site, *Asterias vulgaris* showed a poorly defined yearly reproductive cycle, including a period of gametogenesis from October until March when gametogenesis was complete. Spawning followed during July and continued until August; the resulting spent gonads were then quiescent in terms of gamete production again until October. The "major collections" were made corresponding to Sherman's data; in this way specimens with gonads demonstrating (1) active gametogenesis, (2) completed gametogenesis, and (3) no gametogenesis (spent gonads) were obtained. Such specimens were used to observe changes in the basic histology of the gonad resulting from the activity of the germinal epithelium.

In order to determine that the species used in this study was indeed *Asterias vulgaris* and not *Asterias forbesi*, the set of criteria outlined by Aldrich (1956) (amended by some personal observations) was used. Altogether, in the four collections, a total of 60 specimens was collected; thirty-five of these, which proved to be *Asterias vulgaris,* were used.

Dissection of specimens

Specimens were separately soaked in $MgCl_2$ solution (8% in distilled water) until flaccid. This process relaxes the animal and prevents autotomy during dissection. Two standard measurements commonly used in sea-star taxonomy, *R* and *r*, were recorded for each specimen. *R* is the length from the center of the disc along the aboral surface of the ray to the tip; *r* represents the distance from the center to the edge of the disc in an interradial angle. Each animal was then transferred to fresh sea water and dissected under a Bausch and Lomb Stereo-Zoom microscope in the following fashion. Two major cuts were made along each ray, one aborally through the carinal series of ossicles from the tip of the ray to the center of the disc, and another similar longitudinal cut orally through the center of the roof of the ambulacral groove. This procedure separated the animal into five pieces, each consisting of halves of two adjacent rays, joined together at the interradial angle. All extraneous tissue was cut away from each of these pieces, leaving five interradii, each containing two complete units of the reproductive system. Four interradii were then fixed in Bouin's fluid; the fifth was preserved in 70% ethanol.

Procedures for major collections

Specimens were allowed to stand in Bouin's fluid for periods of up to one month, which thoroughly decalcified them. The five interradii representing each animal were separated into two groups and processed as follows:

Group 1. Two entire interradii were dehydrated in ethanol, embedded in paraffin in a vacuum oven, and sectioned serially either longitudinally or transversely to the axis of the rachis of the gonad (see Observations) at 7 μ to 10μ. One set of sections from each specimen was routinely stained with Mallory's Phosphotungstic Acid Hematoxylin (PTAH) (Lillie, 1965) for general histological observations and to reveal details of muscular and connective tissues. Selected regions of the second set of sections were stained with the following techniques: (a) Gomori's silver stain (Gomori, 1947)—to differentiate "reticular" collagen fibers in various connective tissue components of the reproductive system; (b) Aldehyde Fuchsin (Cameron and Steele, 1959) counterstained with Halmi's stain (1952)—to differentiate between elastic connective tissue and muscle and other connective tissue; and (c) Periodic Acid Schiff (PAS) (Lillie, 1965)—to demonstrate the distribution and abundance of the haemal fluid in the haemal sinus (see Observations). The presence of glycogen and other polysaccharide complexes is indicated by this technique.

Group 2. Gonads in the remaining interradii were used for general observations of shape and form of the gonad. The interradius which had been preserved in 70% ethanol was macerated in KOH solution (2% in distilled water) for periods of 2 to 20 minutes, depending on the toughness of the tissues of the ray, until the ossicles could be seen clearly. This preparation was preserved and used for observations to determine the point of suspension of the gonad.

Electron microscopy

In July, 1972, 6 specimens of *Asterias vulgaris* were collected on the leeward side of the breakwater at the Isles of Shoals. These animals consisted of 2 females and 4 males. The specimens were fixed under field conditions in the following way. The gonads of the 6 animals were removed after each specimen had been relaxed separately in $MgCl_2$ solution. Several blocks of tissue, 3–4 mm thick, taken from various parts of each gonad, were fixed at room temperature for 20–30 minutes in 3% paraformaldehyde-glutaraldehyde (Longo and Anderson, 1969) in sea water. The blocks were then diced into 1 mm cubes of tissue, and fixation was continued in a small refrigerator at 4° C. for 1–2 hours. The tissues were rinsed in 3 20-minute changes of seawater and post-fixed for 2 hours in 1% OsO_4 in seawater, followed by 4 10-minute seawater rinses. Dehydration of the tissues was carried out in ethanol (10 minutes each in 25%, 50%, 70%, 95%, 100% and 100% solutions). The tissues were then transferred to a 10-minute rinse in propylene oxide: 100% ethanol, 1:1, followed by 2 10-minute rinses in 100% propylene oxide, and embedded in Epon 812/Araldite 506 mixture (Mollenhauer, 1963). Silver-gray sections were cut on a Porter-Blum MT-2B ultramicrotome using glass knives and subsequently picked up on 0.25% formvar-coated 200 mesh grids. All sections were stained with both 2% uranyl acetate (30 minutes) and lead citrate (15 minutes) (Reynolds, 1963) and examined on a Philips EM 300 operated at 80 kV.

The results from all these procedures have been combined to provide a detailed account of the morphology and histology of the gonad.

Observations

General morphology of the gonad

In either sex of a normal five-rayed specimen of *Asterias vulgaris,* the reproductive system comprises 10 separate units, two in each ray. Each unit is attached

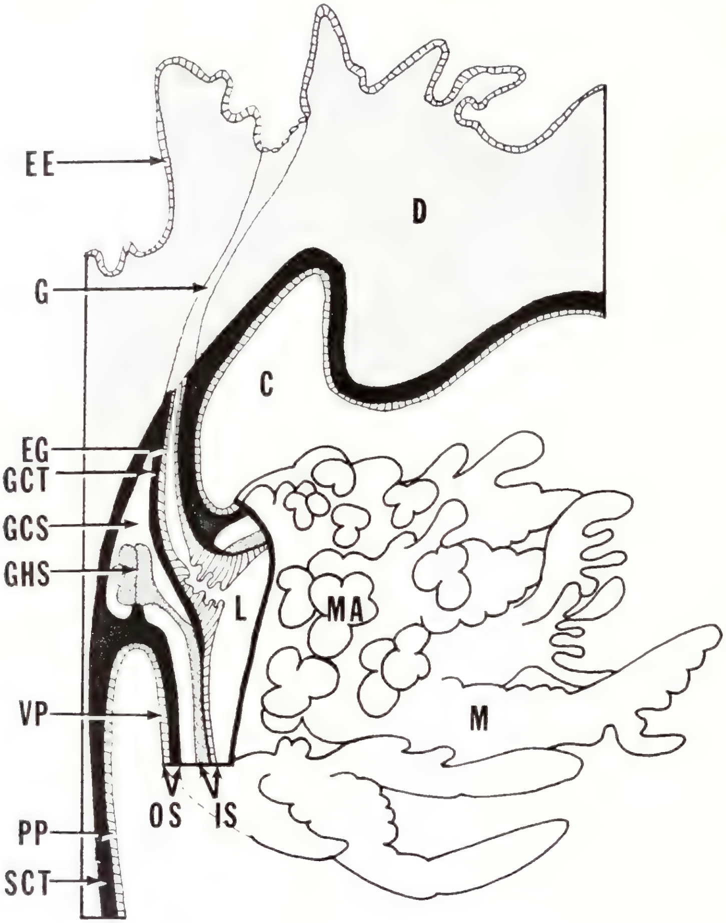

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FIGURE 1. Diagrammatic representation of the inner lateral wall of the ray (on the left side) at the point of attachment of one unit of the reproductive system, showing the relationships of various layers of tissue which make up the gonad with those in the wall of the ray. Symbols used are: C, coelom; D, dermis; EE, external epithelium; EG, epithelium of the gonoduct; G, gonoduct; GCS, genital coelomic (perihaemal) sinus; GCT, gonoduct connective tissue; GHS, genital haemal strand; IS, inner sac; L, lumen of the gonad; M, major acinus; MA, minor acinus; OS, outer sac; PP, parietal peritoneum; SCT, subperitoneal connective tissue; VP, visceral peritoneum.

proximally near the disc on the inner face of the lateral wall of the ray, below the pyloric caecum, and the principal parts of the unit, the gonad, the gonoduct, and the genital haemal strand-genital coelomic (perihaemal) sinus complex, extend to other parts of the ray. The major features of a typical unit are shown diagrammatically in Figure 1. The gonad is large and obvious and projects freely into the coelomic cavity along the length of the ray; the gonoduct and genital

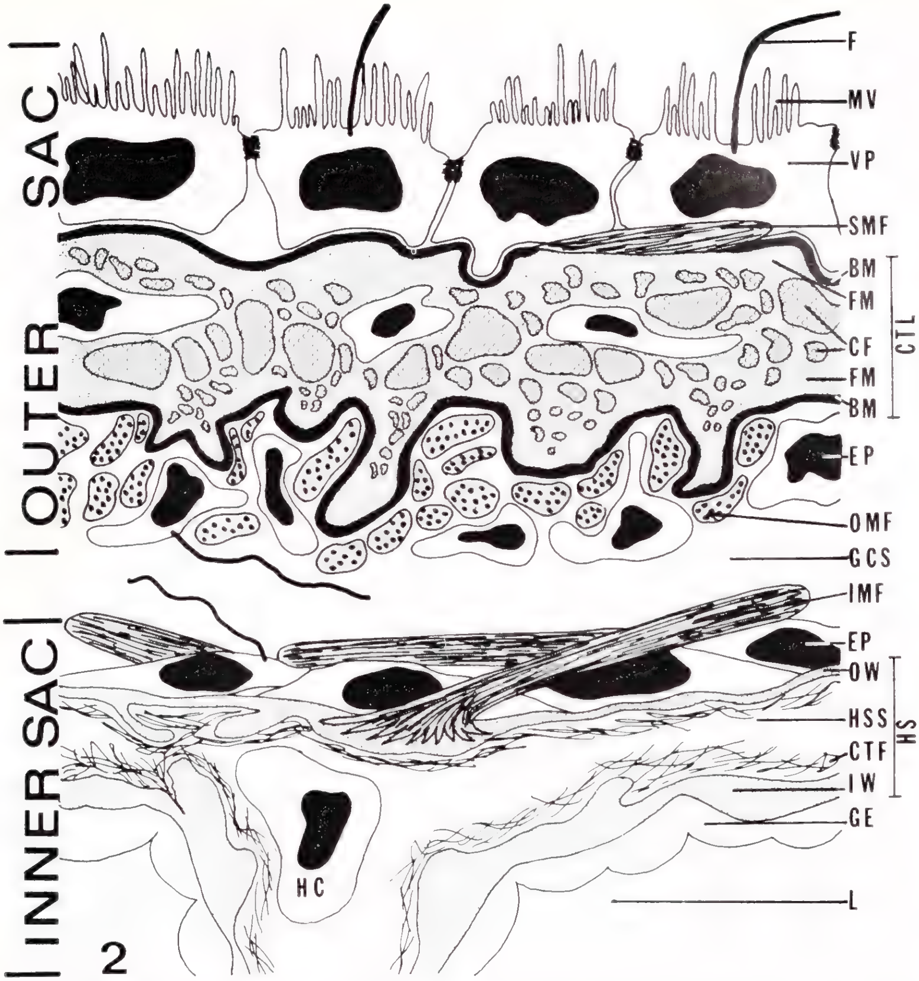
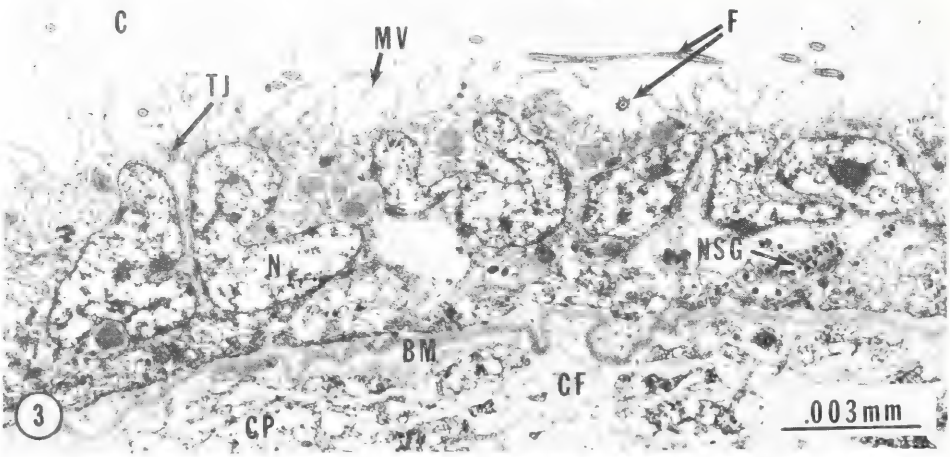


FIGURE 2. Diagrammatic representation of the typical arrangement and appearance of the tissues in the wall of the gonad. Symbols used are: BM, basement membrane; CF, collagen fibers; CTF, connective tissue fibers; CTL, connective tissue layer; EP, epithelium; F, flagellum; FM, fibrous matrix; GE, germinal epithelium; GCS, genital coelomic (perihaemal) sinus; HC, cell in the haemal sinus; HS, haemal sinus; HSS, haemal sinus space; IMF, inner longitudinal muscle fibers; IW, inner wall of haemal sinus; L, lumen of the gonad; MV, microvilli: OMF, outer circular muscle fibers; OW, outer wall of haemal sinus; SMF, subperitoneal muscle fibers; VP, visceral peritoneum.

haemal strand (and its associated coelom) are microscopic, the former penetrating the wall of the ray and opening aborally by several gonopores in the epithelium of the interradius, the latter connecting with the aboral haemal and coelomic rings in the disc.

In both sexes, the gonad is a single large bag consisting of a number of parts. A major piece, the rachis, forms the axis of the gonad; by this piece the gonad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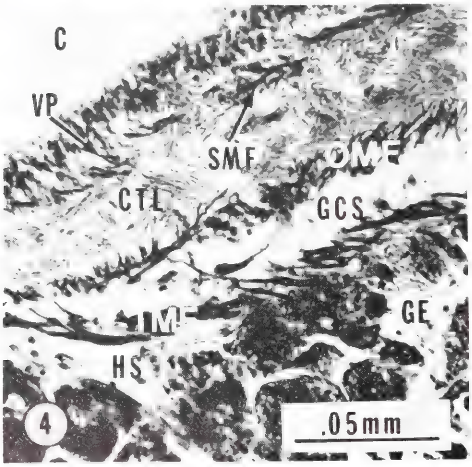
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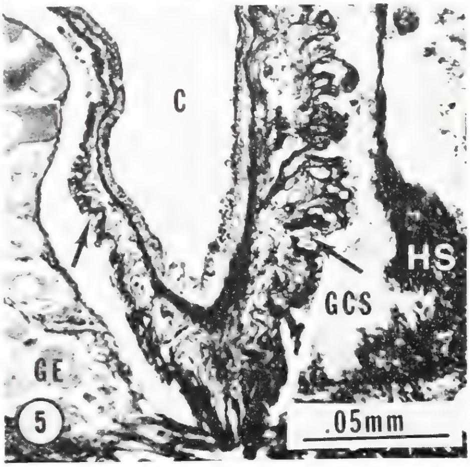
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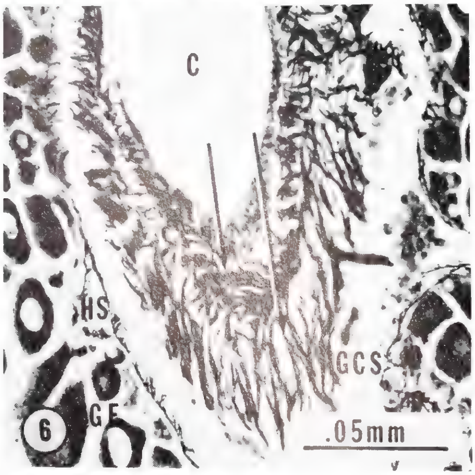
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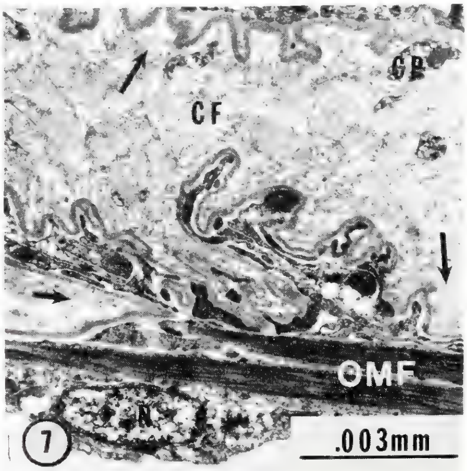
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is suspended from the body wall through a single stalk of connective tissue above the supra-marginal ossicles of the ray. Many projections extend from this axial piece into the coelom. A varying number (10–30) of elongate tubules, the major acini, project from the medial margin of the rachis toward the ambulacral plates. The remaining surface of the rachis, and often much of the major acini, are marked by short rounded protuberances, the minor acini.

Histology of the gonad

The wall of the gonad is composed of several layers of tissue arranged in two groups, the outer and inner sacs, details of which are shown in Figure 2. Although these layers are always found in the same order wherever they occur, their relative dimensions and general appearance differ markedly depending on the part of the gonad they form and on the degree of gametogenic activity occurring in the germinal epithelium at the time.

The *outer sac* consists of a single stratum of visceral peritoneal cells that rests on a layer of fibrous connective tissue to which muscle and epithelial cells are attached internally. The *inner sac* of the gonad is often very thin, complex, and difficult to interpret; full understanding of its structure necessitates the use of low magnification electron microscopy. It is composed of the haemal sinus and associated tissues. In this paper, "haemal sinus" refers to a genital branch of the aboral haemal ring found in association with the germinal epithelium of the gonad. The term is used collectively to include (1) the haemal sinus space, which may contain (2) granular PAS-positive haemal fluid and cells, enclosed by (3) two fibrous layers, called the outer and inner walls of the haemal sinus. The outer wall serves externally as a basement membrane for muscle and epithelial cells

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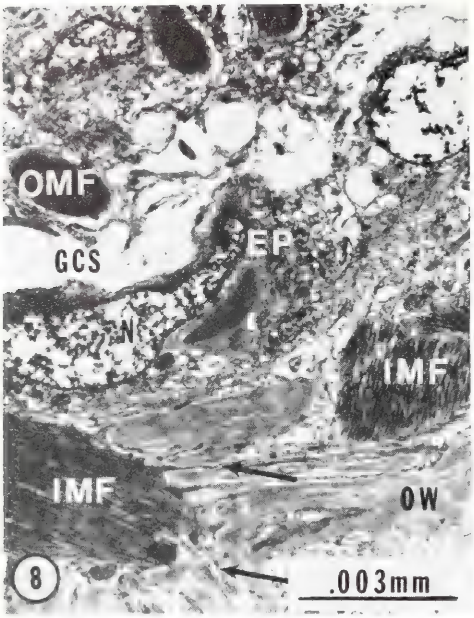
FIGURE 3. Electron micrograph of a section of a testis, showing the cells of the visceral peritoneum; Uranyl acetate and lead citrate. Symbols used are: BM, basement membrane; C, coelom, CF, collagen fibers; CP, cellular process; F, flagellum; MV, microvilli; N, nucleus; NSG, neurosecretory granules; TJ, tight junction.

FIGURE 4. Cross-sectional view of the wall of an ovary to show the general appearance of the tissues that compose it. Compare with Figures 2, 7, and 15. PTAH. Symbols used are: C, coelom; CTL, connective tissue layer; GE, germinal epithelium; GCS, genital coelomic (perihaemal) sinus; HS, haemal sinus; IMF, inner longitudinal muscle fibers; OMF, outer circular muscle fibers; SMF, subperitoneal muscle fibers; VP, visceral peritoneum.

FIGURE 5. A section of the wall of an ovary at the base of a minor acinus where the darkly staining borders of the outer connective tissue layer are obvious; muscle and epithelial cells lie within the folds of the inner border (arrows; lightly stained here); Aldehyde fuchsin, Halmi's stain. Symbols used are: C, coelom; GCS, genital coelomic (perihaemal) sinus; GE, germinal epithelium; HS, haemal sinus.

FIGURE 6. Section of the wall of an ovary at the base of a minor acinus, showing the distribution of the fibers of the outer connective tissue layer (arrows) and the connective tissue fibers of both walls of the haemal sinus near the germinal epithelium; Gomori's silver stain. Symbols used are: C, coelom; GCS, genital coelomic (perihaemal) sinus; GE, germinal epithelium; HS, haemal sinus.

FIGURE 7. Electron micrograph of a section of an ovary showing features of the connective tissue layer of the outer sac of the gonad. Notice, in particular, the wavy basement membranes of the visceral and genital-coelomic peritoneal layers; the arrows indicate the fine fibers of the matrix. Compare with Figure 4; Uranyl acetate and lead citrate. Symbols used are: CF, collagen fibers; CP, cellular processes; N, nucleus of an epithelial cell; OMF outer circular muscle fibers.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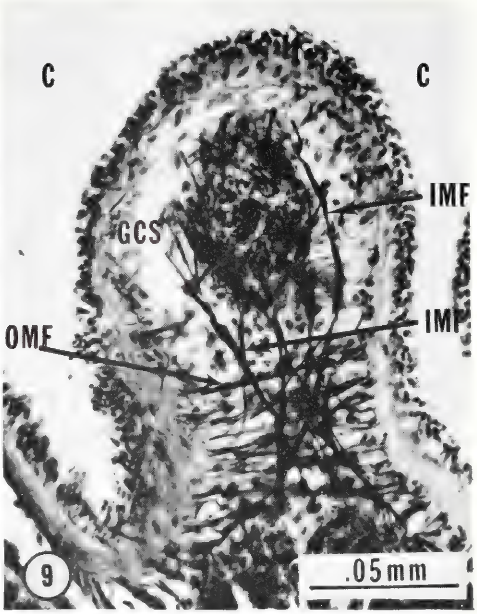
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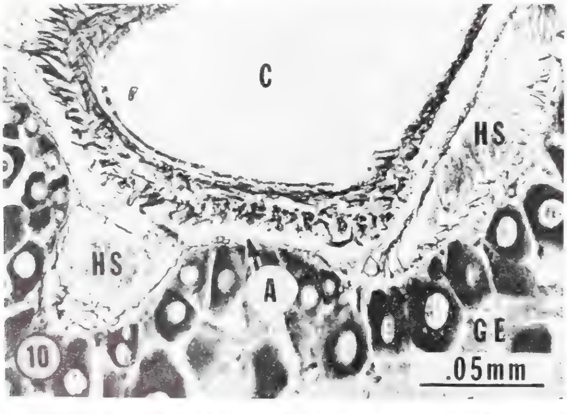
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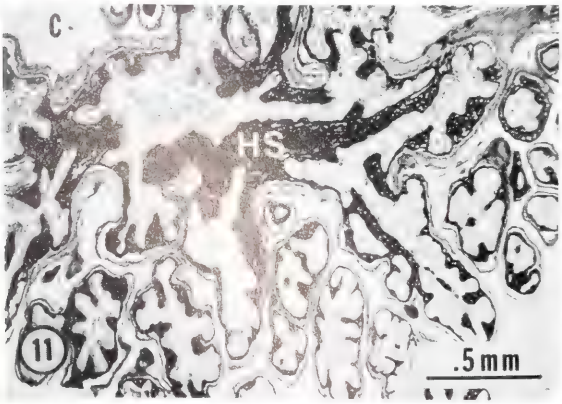
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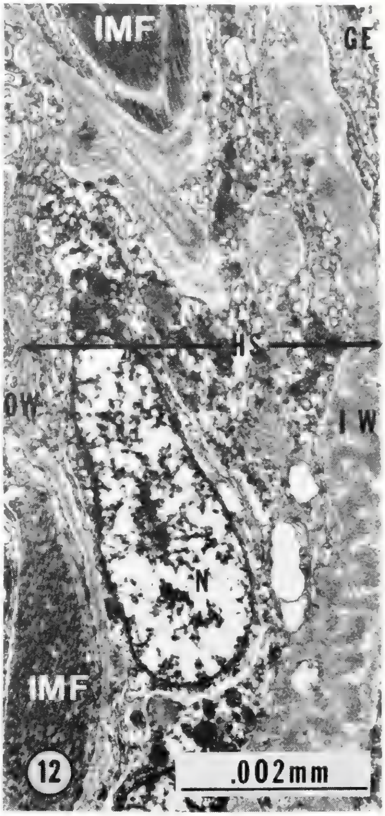
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found in the genital coelomic (perihaemal) sinus; the inner wall serves internally as a basement membrane for the germinal epithelium.

A distinct genital coelomic (perihaemal) sinus lies between the sacs which make up the wall of the gonad (Fig. 2, GCS). This sinus extends through the connective tissue stalk suspending the gonad and in the wall of the ray connects with a genital branch of the aboral coelomic (perihaemal) ring (Fig. 1, GHS). Such an attachment exists for each gonad, and thus all ten gonads are interconnected by way of this coelomic sinus. Both sacs are attached to the connective tissue of the gonoduct near its orifice within the gonad (Fig. 1). Elsewhere, although the two sacs may be in contact and nearly indistinguishable from each other at the limit of resolution of the light microscope, observations utilizing the electron microscope show clearly that they remain physically distinct and maintain their separate identities. In the living animal, a fluid is undoubtedly present in the genital coelomic (perihaemal) sinus, but no trace of it is evident in fixed, sectioned, and stained material. We now proceed to describe specific components of the wall of the gonad in greater detail.

The visceral peritoneal cells of the gonad are directly continuous with those of the parietal peritoneum (Figs. 1, 2, and 4, VP). The concept of these cells which is formed from observations using the light microscope is relatively simple and incomplete, because the cells are quite small and are usually not well preserved. The epithelial cells are basically cuboidal; however, their shape is quite variable and depending upon the degree of expansion of the gonad, the cells may be flat, cubical, or tall and columnar in appearance on various regions of the same gonad. In gonads that have completed gametogenesis, most cells are flat (squamous), while in spent gonads or those involved in gametogenesis, all three types of cells may be found. Observations with the electron microscope reveal additional details (Fig. 3). The cytoplasm of most cells contains few organelles, although a large

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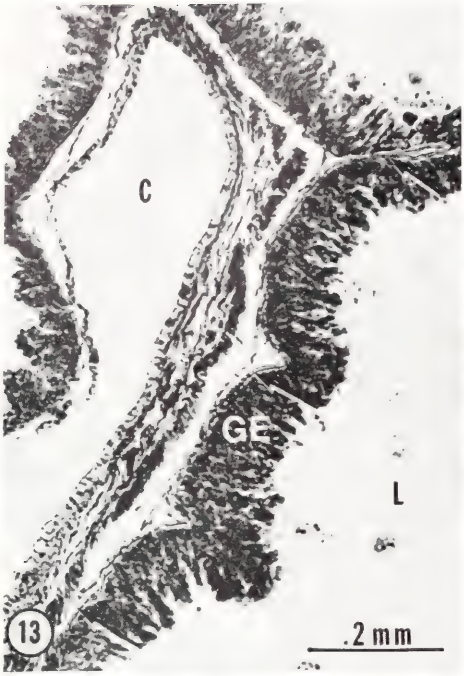
FIGURE 8. Electron micrograph of a section of an ovary, showing muscle fibers of the outer and inner sacs of the gonad. Notice the firm attachment of the inner longitudinal muscle fibers to the outer wall of the haemal sinus (arrows); Uranyl acetate and lead citrate. Symbols used are: EP, epithelium covering the outer wall of the haemal sinus; GCS, genital coelomic (perihaemal) sinus; IMF, inner longitudinal muscle fibers; N, nucleus of epithelial cell; OMF, outer circular muscle fibers; OW, outer wall of haemal sinus.

FIGURE 9. Longitudinal section of one minor acinus from an ovary, showing the orientation of the outer (OMF) and inner (IMF) muscle fibers; PTAH. Symbols used are: C, coelom; GCS, genital coelomic (perihaemal) sinus.

FIGURE 10. Section of an ovary, showing adjacent regions of the haemal sinus which appear either as a jumble of connective tissue fibers (A) or as an expanded space (HS); the connective tissue fibers are embedded on the luminal side of both the outer and inner walls of the sinus; Gomori's silver stain. Symbols used are: C, coelom; GE, germinal epithelium.

FIGURE 11. Several major acini sectioned longitudinally showing the general distribution of the haemal sinus and its PAS-positive contents; PAS. Symbols used are: C, coelom; HS, haemal sinus.

FIGURE 12. Electron micrograph of a section of an ovary, showing the haemal sinus and its components in a region where the walls of the sinus are close together. The haemal sinus space contains cells and not haemal fluid; Uranyl acetate and lead citrate. Symbols used are: GE, germinal epithelium; HS, haemal sinus; IMF, inner longitudinal muscle fibers; IW, inner wall of haemal sinus; N, nucleus of a cell in the haemal sinus; OW, outer wall of haemal sinus.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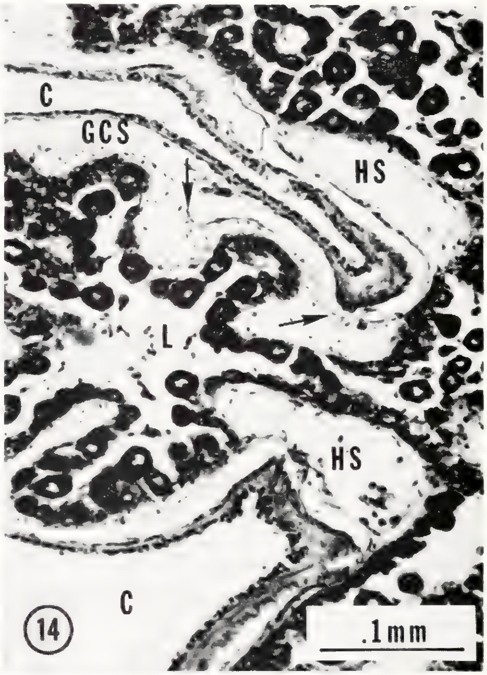
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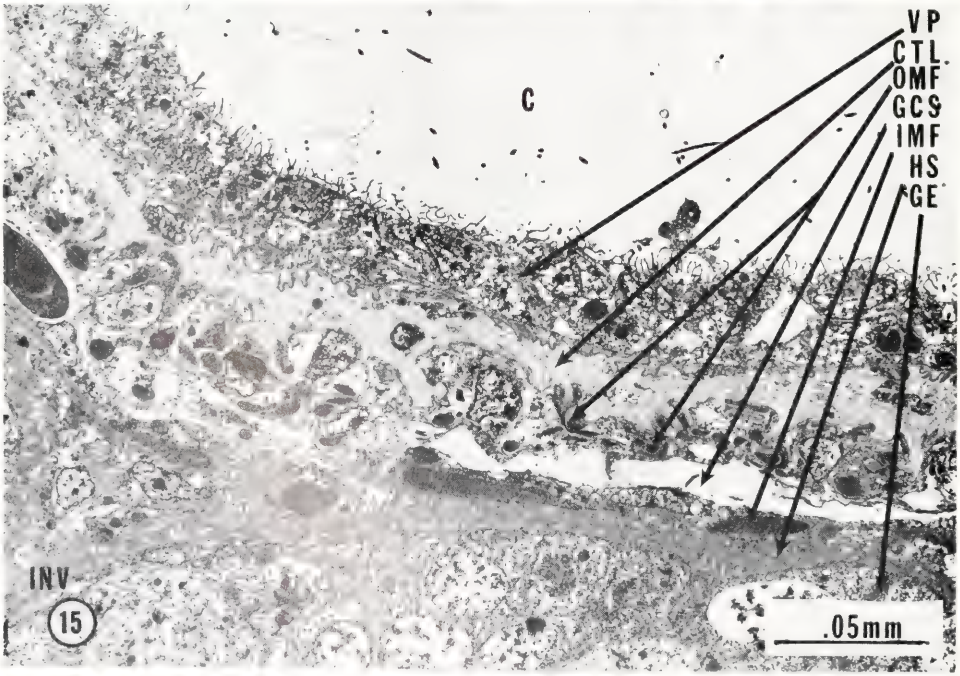
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nucleus, golgi apparatus, mitochondria, flagellar basal bodies, and granules are present. Microvilli surround the base of the single flagellum of each cell as a distinct circle and are also distributed randomly over the free surfaces of cells. The peritoneal cells are attached to each other by tight junctions, often through extremely attenuated processes. Membrane bound granules are found between and below the typical peritoneal cells (Fig. 3, NSG) and are similar to structures identified by Buslé (1969) and Davis (1971) as nerve cell processes.

Fibers which stain yellow with Orange G and blue with PTAH but do not stain with silver are occasionally found in thick regions of the outer sac below the peritoneum surrounding the bases of the major and minor acini (Figs. 2 and 4, SMF). Such fibers may form a network at the base of the peritoneum; preliminary observations with the electron microscope show that they are muscle fibers.

The outer connective tissue layer (Figs. 2 and 4, CTL), is continuous with a similar layer located subperitoneally in the wall of the ray (Fig. 1, SCT). This continuity is evident across the stalk suspending the gonad, which is composed exclusively of tissues corresponding to those of the outer sac of the gonad (Fig. 1). In the animals observed, the maximum thickness of this layer is 30 μ to 35 μ; it is thickest along the wall of the rachis especially around the bases of the major acini and near the point of suspension of the gonad. In other places, as on the distal tip of the gonad or on rounded surfaces of the acini, it may be 2 μ or less in thickness. With the light microscope, the connective tissue layer appears to be condensed on both borders, and these regions stain dark blue with aldehyde fuchsin (Fig. 5). Sandwiched between these two condensed laminae is a thick zone of fibers which stain pink with PTAH (Fig. 4, CTL) and also stain with Gomori's silver technique (Fig. 6, arrows). This layer is quite elastic and resilient even in preserved material. Observations of this connective tissue with the electron microscope (Fig. 7) support and extend the interpretation just presented. The entire layer is composed of a matrix of fine fibers which is often interrupted or replaced by collagen fibers of various sizes running in all directions and by occasional groups of cells or cellular processes. The outer and inner margins of this layer, seen as condensed

FIGURE 13. Section of a testis showing the thin inner wall of the haemal sinus as seen by light microscopy (arrows) and the indistinct nature of the outer wall of the sinus; PTAH. Symbols used are: C, coelom; GE, germinal epithelium; L, lumen of the gonad.

FIGURE 14. Section of a minor acinus of an ovary showing the single cell layer covering the outer wall of the haemal sinus (arrows) (see Figure 8) and the invaginations projecting into the lumen of the gonad formed by the inner wall of the haemal sinus and the germinal epithelium; PTAH. Symbols used are: C, coelom; GCS, genital coelomic (perihaemal) sinus; HS, haemal sinus; L, lumen of the gonad.

FIGURE 15. Electron micrograph of the wall of an ovary taken at low magnification. Using this figure, compare the resolution of details of the various tissue components of the wall of the gonad with that seen in light micrographs of similar subjects, in particular, Figure 4; Uranyl acetate and lead citrate. Symbols used are: C, coelom; CTL, connective tissue layer; GE, germinal epithelium; GCS, genital coelomic (perihaemal) sinus; HS, haemal sinus; IMF, inner longitudinal muscle fibers; INV, invagination of the inner wall of the haemal sinus and the germinal epithelium; OMF, outer circular muscle fibers; VP, visceral peritoneum.

borders with the light microscope, form basement membranes for the visceral peritoneum and muscle and epithelial cells of the genital coelomic (perihaemal) sinus, respectively. These basement membranes are usually wavy in appearance as seen with both the light and electron microscopes (Figs. 5 and 7) and are composed of fine fibers similar to those in the matrix with which they are intimately associated. Complex, sometimes large cells and attenuated cellular processes are found among the fibers of the connective tissue layer; these are assumed to be associated with the formation of fibers.

The muscle fibers attached to the inner basement membrane of the outer sac of the gonad (Figs. 2, 4, 7 and 8, OMF) stain yellow with Orange G, blue with PTAH, and not at all with Gomori's silver stain. These fibers run circularly around the axes of the rachis and acini of the gonad (Fig. 9). Viewed in sections, they often lie close against the connective tissue layer, within folds formed by this layer (Figs. 2, 5 and 7).

Observations with the electron microscope indicate that a discontinuous epithelium lines the genital coelomic (perihaemal) sinus throughout the gonad (Figs. 2, 7, and 8). The cells of this epithelium occur among muscle fibers of the outer and inner sacs; they are often amoeboid and many are flagellated. Membrane bound granules associated with the epithelial cells of the genital coelomic (perihaemal) sinus are similar to those seen in several sea-stars by Davis (1971).

While all these tissues of the outer sac of the gonad may vary in thickness and shape, the tissues of the inner sac are even more variable; their appearance depends upon the activity of the germinal epithelium. Muscle fibers are often numerous in association with the outer wall of the haemal sinus only (Figs. 2, 4, and 8, IMF); they are intimately connected to this wall externally (Fig. 8, arrows). Such fibers are oriented longitudinally along the axes of the rachis and acini forming a basket of muscle fibers (Fig. 9).

Tissues associated with the haemal sinus of the gonad are similar to those of the genital haemal strand with which the sinus is continuous near the gonoduct (Fig. 1). Through the light microscope, the sinus appears either as a single layer or often in adjacent regions as two layers separated by the haemal sinus space. These layers are quite indistinct but correspond to the walls of the haemal sinus. Silver staining reveals distinct, thin, connective tissue fibers (clearly collagen fibers as seen with the electron microscope) running in random directions on the luminal side of both walls (Fig. 10). At certain times of the year (in this study, October and July, in particular) separate channels of the haemal sinus expand and become continuous; the sinus space is filled with PAS-positive haemal fluid evident in sectioned and stained material as a reddish-pink coagulate (Fig. 11, HS). Details of the haemal sinus are resolvable with the electron microscope. Only with the high resolution provided by this instrument can it be appreciated that both walls of the haemal sinus are present throughout the gonad; they are often wavy in appearance and may send projections into the lumen of the haemal sinus (Fig. 12). Each wall is composed of a fine fibrous material similar in appearance to the matrix portion of the connective tissue layer of the outer sac. Except in very unusual cases, only the thin inner wall of the haemal sinus is discernable with the light microscope (Fig. 13, arrows), although, when the sinus is maximally expanded, the existence of the outer wall is suggested by the single layer of epithelial cells which covers it externally (Figs. 2 and 8, EP and 14, arrows). Both walls of the sinus may stain brightly with PAS and aldehyde fuchsin. The inner wall of the haemal sinus and its associated germinal epithelium often form deep folds that project into the lumen of the gonad (Figs. 2, 4, 10, 11, 13, 14 and 15). In sections these may appear triangular or they may have various shapes; similar invaginations were noticed by Mauzey (1966) in *Pisaster* and Chia (1968) in *Leptasterias*. The haemal sinus space generally contains granular fluid in which collagen and other fine fibers are often apparent; the orientation of these fibers may be random or may parallel the walls of the sinus. Cells with various shapes and contents occur throughout the sinus space (Figs. 12 and 15); some are amoeboid with long cytoplasmic processes and others are contracted and filled with granules.

In mature animals, the germinal epithelium forms a continuous lining of the lumen of the gonad; it connects with the epithelium of the gonoduct (Fig. 1, EG). The germinal epithelium is composed of follicular cells, oogonia, and oocytes in females and spermatogonia, spermatocytes, and maturing spermatozoa in males. Of all the tissues mentioned, seasonal changes are most dynamic in the germinal epithelium in connection with its yearly production of gametes. These changes have been described by a number of authors for several sea-stars and the essentially similar changes seen in *Asterias vulgaris* will not be discussed here.

Discussion

The observations just concluded provide a detailed description of the gonad of *Asterias vulgaris* in morphological and histological terms; morphologically the gonads of this species differ from those seen in most other sea-stars, histologically they appear to be quite similar to those in any asteroid. From the standpoint of microscopic anatomy it is fortunate that descriptive studies of various aspects of the histology of the gonads of asteroids are numerous. Collectively these provide a good outline which is applicable in general to almost any sea-star. As mentioned earlier, however, no studies are available which give a detailed description of the tissues that make up the wall of the gonad in both sexes of any species based on observations using both the light and electron microscopes. Many features of the general organization of various tissue layers of the gonad are obvious only with the light microscope, while details of the histology of the tissues are obvious only with low magnification electron microscopy. In the following section, the histology of the wall of the gonad of *Asterias vulgaris* will be compared with corresponding features of the gonads of other sea-stars which have been previously described.

The visceral peritoneum of the outer sac of the gonad has been described by some authors as a single layer of ciliated cuboidal cells (Field, *Asterias vulgaris*, 1892; Gemmill, *Solaster endeca*, 1912; and Chia, *Leptasterias hexactis* and *Henricia sanguinolenta*, 1968, 1970). In contrast, some observers state that it consists of ciliated flat cells (Ludwig and Hamann, 1899, and Davis, 1971). From the present study, it is apparent that this layer is basically composed of simple flagellated cuboidal cells like those which make up the peritoneum elsewhere. Local pressures and stresses result in their being stretched thin over protuberances or laterally compressed in folds and angles. Preliminary observations on the ultrastructure of the peritoneal cells in the present study are in general agreement with those of Tangapregassom and Delavault (1967) and Davis (1971). Buslé (1969) and Davis (1971) indicate that nerve cell processes containing granules that are possibly neurosecretory in nature are often seen below and between the peritoneal cells of the gonads of the sea-stars with which they worked. Granules were observed in the present study in a similar relationship with the visceral peritoneum, but the evidence is insufficient to confirm identification of such granules with nervous tissue in *Asterias vulgaris.*

The muscle fibers seen in association with the peritoneum of the gonad in *Asterias vulgaris* have never been described in other species. Their function in the gonads of this species is unknown, although they might cause local deformations of the peritoneum that direct movement of coelomic fluid over the surface of the gonad.

Hoffmann (1872), in his studies of *Asterias rubens,* was the first investigator to mention the connective tissue of the outer sac of the gonad, describing it as composed of delicate homogeneous connective tissue. More detail is presented by Gemmill (1914), who notes that in *Asterias rubens* the outer connective tissue layer may be subdivided into superficial and deeper sheets. Tangapregassom and Delavault (1967), Buslé (1969), and Davis (1971) state that it is composed of collagen fibers in which there are occasional pockets of cells; other investigators mention that it exists but give no details. It is apparent from observations described earlier that this layer is extremely tough and elastic in *Asterias vulgaris.* Its borders serve as basement membranes, stain brightly with aldehyde fuchsin, are usually wavy in sections, and are very likely similar to the elastic tissue of vertebrates. The elastic basement membranes of this layer allow the gonad to expand while the abundant collagen fibers found between them maintain its basic shape.

The circular muscle fibers facing the genital coelomic (perihaemal) sinus on the inside of the outer connective tissue layer are similar to the circular fibers described by Hoffmann (1872) for *Asterias rubens* and by Gemmill (1912) in the ovary of *Solaster endeca.* Hayashi (1935), Chia (1968), Tangapregassom and Delavault (1967), Buslé (1969), and Davis (1971), all recognize muscle fibers in a similar location in the gonads of the various species with which they worked, but according to Hayashi (1935), quoting from the observations of Ohshima (1925), this layer is not present in the testes of *Asterina batheri.* Contraction of these muscle fibers is probably important during the shedding of gametes.

The epithelial cells which line the genital coelomic (perihaemal) sinus of the gonad are very likely related to those in the wall of the ray which line the genital branch of the aboral coelomic (perihaemal) ring; they are flagellated and probably keep the fluid contents of the genital coelomic (perihaemal) sinus in motion.

Longitudinal muscle fibers were described by Gemmill (1912) on the inner sac of the gonad of *Solaster endeca.* Hayashi (1935), Chia (1968), Tangapregassom and Delavault (1967), Bruslé (1969), and Davis (1971) also mention muscle fibers, similarly located, in the gonads of various species, without specifying their orientation. In *Asterias vulgaris,* as mentioned, longitudinal muscle fibers are attached only to the outer wall of the haemal sinus facing the genital coelomic (perihaemal) sinus. The function of these fibers remains unknown, although by their contraction they may exercise some control over the volume of fluid contained in the haemal sinus.

Although most previous investigators have mentioned the presence of tissues associated with the haemal sinus, none has given a detailed description of the components of the sinus. Taken together, the works of Gemmill (1914) and Davis (1971) give the most complete account. Gemmill indicates that a branch of the genital haemal ring connects with the inner sac of the gonad near the gonoduct and then branches irregularly over the surface of this sac; Davis describes the haemal sinus as a space filled with granular contents, cells, and collagen fibers, surrounded by two laminae. From the present study, it is clear that the sinus is an expansible bag with walls that are possibly elastic in character; the extreme variability in form and appearance of the haemal sinus is closely associated with changes in the condition of the germinal epithelium. In gonads which have completed gametogenesis, the sinus is nearly empty, its walls are pressed together, and the haemal sinus space is occluded. Alternatively, in gonads which are either involved in gametogenesis or have spawned recently, the haemal sinus may be filled with fluid and cells and its walls are widely separated. Work by Mauzey (1966) and Chia (1968) has shown that haemal fluid is strongly PAS-positive. Mauzey (1966) finds that the haemal fluid in *Pisaster ochraceus* does not contain glycogen since haemal fluid is PAS-positive before and after salivary amylase treatment. Chia concludes that this fluid may store and supply nutrients to the developing germinal cells, citing as evidence its location and the time during the reproductive cycle when it becomes conspicuous. In the present study, haemal fluid was present in greater volume in females than in males; it was found while gametes were maturing and was very abundant after gametes had been shed. No evidence is available on the function of this fluid in *Asterias vulgaris.*

Through the aboral haemal ring and its genital branches, the haemal sinus of each gonad is connected with those found in all 9 other gonads and also indirectly to other organ systems in the body: the pyloric caeca, axial organ, radial nerves of the rays, etc. Such a system of channels between the reproductive organs and other organ systems is no doubt important in transmitting fluids and other materials to and from the gonads, and also in coordinating the activities of all 10 gonads during the annual reproductive cycle.

From every aspect, the studies reported here reveal the limited amount of detailed information upon which our understanding of structure and function in the gonads of asteroids is based. It is thus obvious that additional studies, utilizing as many and varied species as possible, are highly desirable.

I wish to express my gratitude to Dr. John M. Anderson for his careful guidance throughout the course of this study and for his meticulous editorial assistance in the preparation of this manuscript. I also thank my wife, Wilise, for her valuable assistance and encouragement.

SUMMARY

The results reported here provide a detailed account of the general morphology and histology of the male and female gonads of the sea-star *Asterias vulgaris*. The reproductive system of this sea-star (normal five-rayed specimens) consists of 10 separate units, each located proximally on the lateral wall of the ray, one on either side of the ray. Each unit is composed of a gonad, a gonoduct, and genital branches of the aboral haemal and coelomic rings. The gonad is a single bag-like structure with several protrusions (termed major and minor acini) extending from its surface. Its wall is composed of two sacs, one inside the other, separated by the genital coelomic (perihaemal) sinus. The outer sac consists of visceral peritoneum, an elastic-collagenous connective tissue layer, and many epithelial cells and circular muscle fibers. The inner sac comprises epithelial cells and longitudinal muscle fibers, the haemal sinus and contents, and germinal epithelium. The haemal sinus includes the haemal sinus space, filled with granular haemal fluid, cells, and collagen and other fine fibers enclosed by two fibrous laminae. Significant modifications in the form of the gonad and in the condition and relationships of the tissues which compose it occur during the annual reproductive cycle. Both sacs are stretched during growth of the gonad, the outer layers becoming attenuated and the inner layers being pressed against the outer, often obliterating the genital coelomic (perihaemal) sinus. The inner group of tissues is often extensively folded, pushing ridges formed from the inner wall of the haemal sinus and germinal epithelium into the lumen of the gonad.

It is pointed out that previous studies on the gonads of asteroids have been relatively few, with no study for any species dealing comprehensively with morphological and histological details of the gonad based on both light and electron microscopy. Comparison of the results of the present study with observations of previous investigators indicates that although significant differences occur (especially in morphological terms), the general features of the histology of the wall of the gonad of many sea-stars are similar. In order to broaden our base for comparative studies, and to pursue significant problems, morphological, histological, histochemical, and ultrastructural investigations should be extended to as many asteroid species as possible.

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THE OCCURRENCE, DISTRIBUTION AND ATTACHMENT OF THE PEDUNCULATE BARNACLE *OCTOLASMIS MÜLLERI* (COKER) ON THE GILLS OF CRABS, PARTICULARLY THE BLUE CRAB, *CALLINECTES SAPIDUS* RATHBUN[1]

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During the summer of 1973 crabs were collected regularly from Beaufort Inlet, North Carolina and examined for the presence of the epizoic barnacle, *Octolasmis mülleri* (Coker), within the branchial chambers. The blue crab, *Callinectes sapidus* Rathbun is the most common crab in Beaufort Inlet (Dudley and Judy, 1971) and was therefore chosen as the host for a detailed study of *O. mülleri.* The life-history and migrations of *C. sapidus* (Van Engel, 1958; Cargo, 1958; Costlow and Bookhout, 1959; Tagatz, 1968; Judy and Dudley, 1970; Dudley and Judy, 1971) have been studied because this crab is exploited commercially. The migrations of this crab may also influence the life-histories of epizoites. The present study investigates the occurrence, distribution and mode of attachment of the epizoic *O. mülleri* on the gills of *C. sapidus* and discusses the possible correlations with the life cycle and habits of the crab.

MATERIALS AND METHODS

When barnacles were found to be present in a blue crab, all the gills were removed and the number and position of the barnacles on each gill noted.

During July several blue crabs were collected which had only the recently settled cypris larva of the barnacle present on the gills. These larvae, together with the portion of the gill to which they were attached, were fixed for a histological study. Adult specimens of *O. mülleri* were also fixed in Carnoy's and Bouin's fixatives for a histological study of the cement apparatus. Wax sections (5 μ thick) were stained with Heidenhain's azan. The peduncles of both *O. mülleri* and the related *Lepas anatifera* were fixed in 1% OsO_4 in veronal buffer, dehydrated and embedded in Araldite. One μ thick transverse sections of the peduncles stained with toluidine blue in borax (1% aqueous solution) were examined with the light microscope.

RESULTS

The 4th gill of *C. sapidus* is drawn diagrammatically in Figure 1a. It is composed of many paired platelets (pl.) which have well defined spines and knobs around their outer margin (Fig. 1b). The larger efferent blood vessel (e.v.) and the smaller afferent vessel (a.v.) are situated on the inner and outer side of the

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[1] This study was carried out at the Duke Marine Laboratory, Beaufort, N.C. 28516.

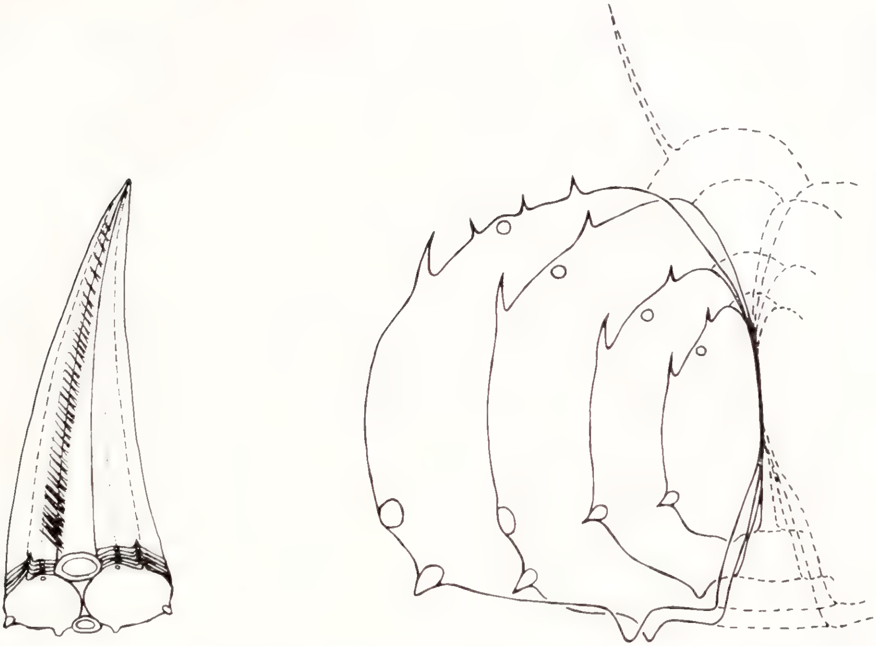


Figure 1. (a) A drawing of the 4th gill of *Callinectes sapidus* showing the paired platelets (pl.), small afferent vessel (a.v.) and larger efferent vessel (e.v.); (b) drawings of gill platelets taken from different regions (1, 2, 3, 4) of the same gill. The platelets are divided into afferent (aff.) and efferent (eff.) zones and show the marginal projections: spines a, b, d, g and knobs c and e; a.v., afferent vessel; e.v., efferent vessel; sp., spine.

gill respectively. Most of the marginal projections of the platelets emerge at an angle to the surface of the platelet itself and some of these presumably act as "spacers" keeping the platelets separated and allowing water circulation. The spines a, b, d, f and g (Fig. 1b) can be picked out with the naked eye, but the more interior knobs c and e are hidden from view. In the proximal region of a gill the platelets possess all the spines and knobs previously mentioned, but spines g and knob c disappear towards the distal end. Seven of the eight pairs of gills in the crab lie close to one another so that platelets of adjacent gills touch. Each platelet can be divided equally into afferent and efferent zones (Fig. 1b). Examina-

Table I

Variation in the barnacles settled on each of 8 pairs of gills from 25 blue crabs.

Source of variation	Degrees of freedom	Mean square	F-ratio
Between gills	7	0.1961	147
Within gills	192	0.00133	
Total	199		

tion of 400 gills (25 blue crabs) revealed that 83.06% of the barnacle population occurred on the efferent side and 16.94% on the afferent side. The barnacle population on the efferent side of the gills was analysed in more detail. The mean number of *O. mülleri* $n_1, n_2 \ldots n_8$ was assessed on each of the eight pairs of gills of 25 crabs and the proportion

$$p_x = \frac{n_x}{\sum_{x=1}^{x=8} n_x}$$

calculated for each gill pair. Analyses of variances was carried out on the ratios $p_1 \ldots p_8$ for the 25 crabs. The results presented in Table I show that the variation between the mean proportionate infestation rate on the eight different pairs of gills exceeded significantly the variation between replicates ($F = 147$, $P < 0.001$). It

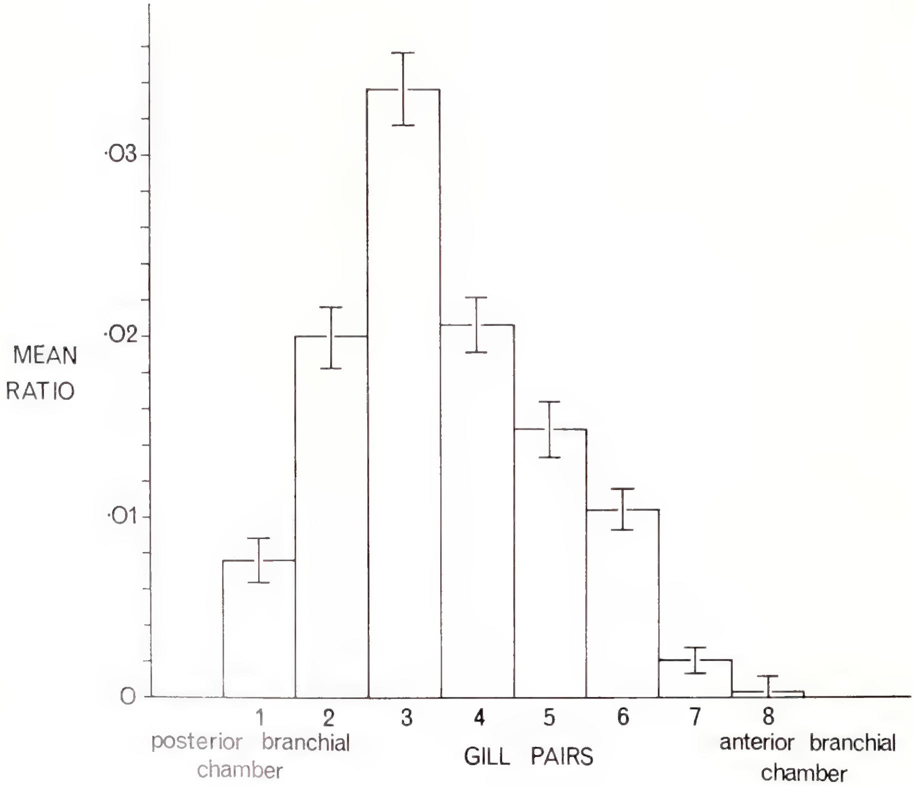


FIGURE 2. Histogram showing the mean ratios $\left[\frac{\text{No. of barnacles on gill pair}}{\text{Total No. barnacles on crab}}\right]$ of barnacles on the different gills of *C. sapidus*. The vertical bars indicate the fiducial limits at the 95% level of significance.

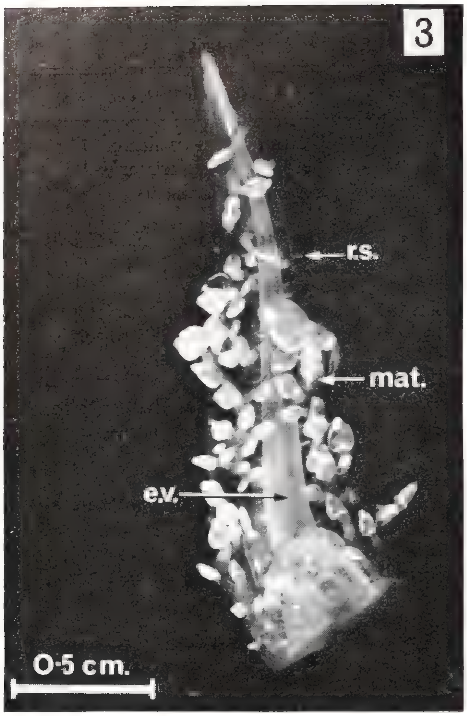
TABLE II

List of crabs on which Octolasmis mülleri *has been found.*

Crab	Reference	Habitat (from Williams, 1965)
**Arenaeus cribrarius* Lamarck		Ocean. Close to shore. Seldom enters estuaries. Shore—37 fathoms.
Calappa flammea Herbst	Humes, 1941.	Ocean. Surface—40 fathoms.
Callinectes ornatus Ordway	DeTurk, 1940; Humes, 1941.	Coastal. Often estuaries, sometimes to fresh water. Surface—40 fathoms.
**Callinectes sapidus* Rathbun	Coker, 1902; Pilsbry, 1907; Scarff, 1966; Scrocco & Fabianek, 1969.	Shallow ocean. Estuaries to fresh water. Surface—20 fathoms.
**Hepatus epheliticus* L.	DeTurk, 1940; Humes, 1941.	Ocean, Sometimes estuaries. 2–25 fathoms.
Libinia dubia H. Milne Edwards	DeTurk, 1940.	Ocean. Shore—25 fathoms.
**Libinia emarginata* Leach	DeTurk, 1940; Coker, 1902.	Ocean. Shore—27 fathoms, occasionally 68 fathoms.
**Menippe mercenaria* Say	Coker, 1902; Humes, 1941; Pearse, 1947.	Ocean and estuary. Surface—28 fathoms.
**Ovalipes ocellatus* Herbst		Ocean, close to shore. Surface—18 fathoms.
Panopeus herbstii H. Milne Edwards	Humes, 1941	Coastal. Often estuaries. Surface—12 fathoms.
**Persephona punctata aquilonaris* Rathbun		Ocean. 2–30 fathoms.
**Portunus spinimanus* Latreille	DeTurk, 1940.	Ocean. Surface—50 fathoms.
Uca minax Le Conte	Pearse, 1936.	Marshes. Some distance from high salinity water.

* Crabs examined in the present study.

should be noted that the procedure adopted eliminated the influence of differences in individual infestation rate between crabs. Comparison between the mean ratios of the barnacles on the gills is shown graphically in Figure 2. It can be seen that the largest number of barnacles is found attached to gill 3, the numbers decreasing progressively on either side of gill 3.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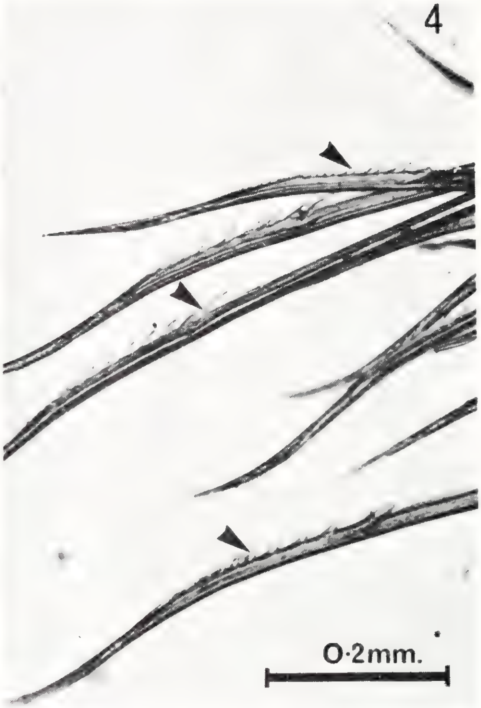
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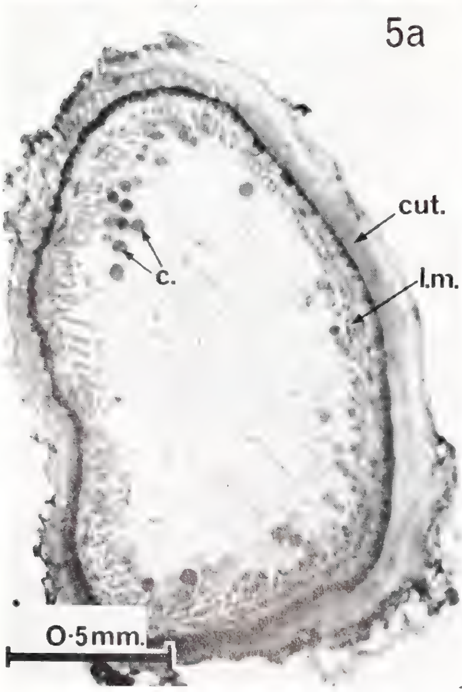
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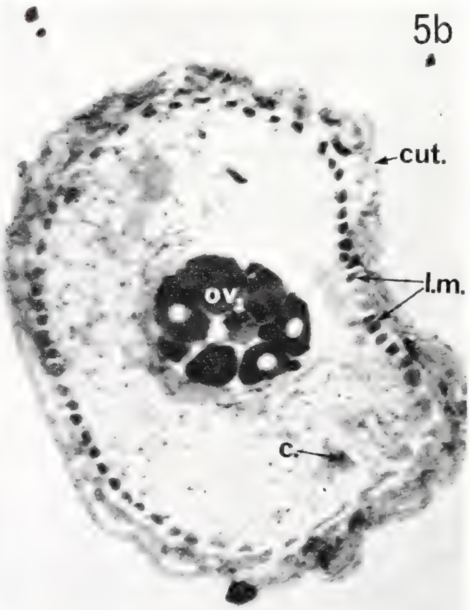
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The efferent zone was split up into several regions (Fig. 1b) and the number of barnacles in these regions counted. Out of a total of 2,953 barnacles counted 1270 (43%) were found in the region between knob e and the efferent vessel, 701 (24%) were associated with spine d, 497 (17%) were associated with knob e, 344 (12%) were found in the region between knob e and spine d and 141 (5%) on the efferent vessel itself.

Cyprids enter the branchial chamber in the inhalant respiratory current of the blue crab. They pass into the hypobranchial side of the branchial chamber. Since the gills are closely opposed to one another the cyprids are unable to pass through to the hyperbranchial side; the gills are therefore a filter to the cyprids. The cyprids must be able to adhere temporarily to the gills whilst searching for a suitable site of attachment, for periodically the respiratory current is reversed by the crab. *O. mülleri* not only settles on a wide variety of crabs (see Table II), but also on different sized crabs within each species (different age groups). In all juvenile crabs moulting is frequent and barnacles which have settled will be shed with the exuvia before reaching maturity; once outside of the host *O. mülleri* is thought to perish. The successful life cycle of *O. mülleri* therefore takes place either in crabs which will no longer moult *i.e.* mated female blue crabs, or on mature crabs which although able to moult still go through prolonged intermoult periods. A large barnacle population on any one crab is composed of individuals ranging from recently settled larvae through to mature adults, Figure 3, showing that the barnacle has a long settling season in comparison with its life span between metamorphosis and maturity.

In addition to withstanding reversal of the respiratory current the cyprids must also survive the cleaning action of the epipodites of the second and third maxillipeds (see Pyle and Cronin, 1950). These epipodites have a fringing mass of setae which have back-curved hooks close to their tips (Fig. 4). The effectiveness of these cleaning appendages on exposed surfaces is reflected in the small numbers of *O. mülleri* which settle on the efferent vessel itself. Settlement on this vessel is restricted to the extreme tip and base of the gill, the areas not successfully cleaned by the epipodites. Judging by the high level of barnacle settlement another part of the platelets perhaps not effectively cleaned is that between the efferent vessel and knob e.

When only a few barnacles were found in a crab they were always settled on the gills. However when a high infestation was found the barnacles were also settled on the wall of the branchial chamber, cleaning appendages and even on the outside of the crab on the basal parts of the maxillipeds. *O. mülleri* was also

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FIGURE 3. An excised gill of *C. sapidus* showing a large number of *O. mülleri* on the efferent side. The barnacles range from mature individuals (mat.) to small recently settled individuals (r.s.); ev, efferent vessel.

FIGURE 4. The tips of some setae from the epipodite of the third maxilliped of *C. sapidus*, showing the back-curved hooks (arrows).

FIGURE 5. Transverse sections through the peduncles of (a) *Lepas antifera* and (b) *Octolasmis mülleri*. In *Lepas* the outer cuticle (cut.) is thick and underlying longitudinal muscles extensive (1 m.); cement cells (c.) are also present in the section. In *Octolasmis* the outer cuticle is much thinner (cut.) and the longitudinal muscles are reduced to a single band (1 m.); cement cells (c.) are also present as is part of the ovary (ov.)

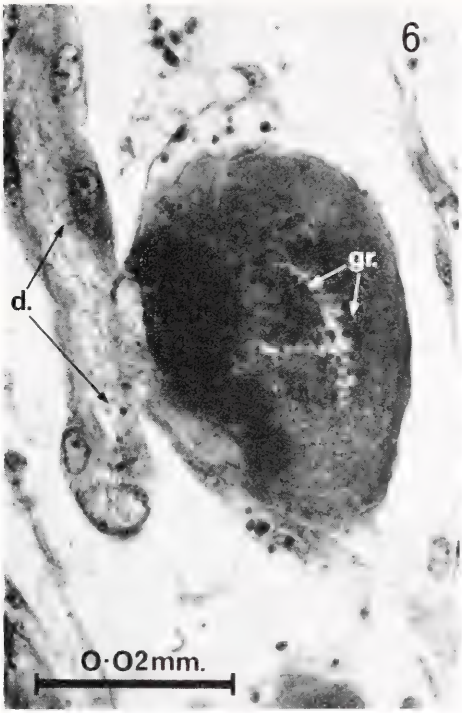
occasionally found settled either on the peduncle or capitulum of other settled individuals under severely crowded conditions.

The movements and behavior of the cyprids in the branchial chambers prior to settlement are not known, but since some site selection occurs active searching probably takes place. Such behavior is common in cirripedes at settlement (see Crisp, 1974).

Although the majority of barnacles are found on the efferent side of the gills of blue crabs some barnacles were found on the afferent side and had presumably been taken into the hyperbranchial side of the branchial chamber as cyprids during the reversal of respiratory current by the crab. This reversal of respiratory current helps cleanse the branchial chamber of debris which has accumulated during the normal respiratory flow. It is possible that as the barnacle population on the efferent side of the gills increases, the cleaning appendages are unable to work effectively and debris accumulates. The host, therefore, reverses the respiratory current more frequently in order to remove the accumulated debris and in doing this takes more cyprids into the hyperbranchial side of the branchial chamber. It was interesting to find that all *O. mülleri* were settled on the afferent side of the gills in the branchial chambers of the crab *Ovalipes ocellatus*. The habits of *O. ocellatus* are different from those of *C. sapidus* in that it spends a larger proportion of time buried in sand. The normal respiratory flow of *O. ocellatus* is equivalent to the reversed respiratory flow of *C. sapidus* (see Pearse, Humm and Wharton, 1942).

Cyprids finally cement themselves to the gills a little way in from the margin of the platelets, both antennules being cemented to the same platelet by secretion from the cement glands within the cypris body (see Walker, 1971). There is apparently no preference shown by the cyprids for the upper or lower side of the platelets at settlement. Following permanent attachment the antennules are flexed thus drawing the body of the cyprid down onto the edges of the platelets. Metamorphosis then follows and the young adult *O. mülleri* emerges. The adult cement apparatus develops (see Walker, 1973) and as the young barnacle grows "adult" cement is laid down. This will initially fill between the two platelets where the cyprid settled, but later as more cement is produced it flows over and between neighboring platelets.

The adult stalk and cement apparatus of the sheltered *O. mülleri* can be compared with that of the related exposed lepad, *Lepas anatifera* (Lacombe and Liguori, 1969). Differences in structure are revealed in traverse sections of the peduncles of these two barnacles (Fig. 5a, 5b). The peduncular cuticle of *O. mülleri* is much thinner than that of *L. anatifera* and the underlying musculature is also much reduced. In *L. anatifera* all the cement cells are aggregated close to the capitulum-peduncle junction and are interconnected by secondary cement ducts. These secondary ducts, which are not lined with chitin, join together eventually forming the two primary ducts (chitin lined), which lead down the peduncle and open out at the ends of the antennules. In *O. mülleri* the cement cells are found throughout the length of the peduncle [*c.f. Pollicipes* (Koehler, 1889)], although the majority of the cells are found in the upper peduncular region. The two primary ducts extend up the peduncle for about two-thirds of its length



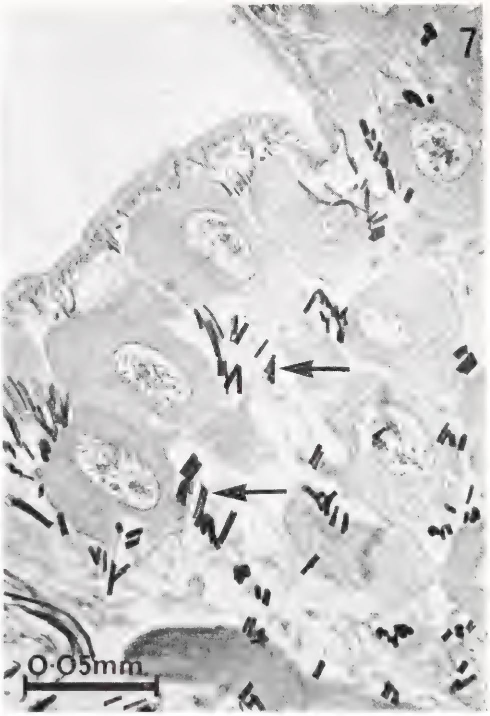


FIGURE 6. A cement cell of *O. mülleri* showing the connection with a collecting duct (d.) The dark mass is secretion which is aggregated around an intracellular canal; gr., granules; Azan.

FIGURE 7. Oblique muscles (arrows) between the cement cells of *Lepas anatifera;* Diazo-coupling for tyrosine.

and lead into the secondary ducts which connect with the cells (collecting ducts).

The cement cells of *O. mülleri* are about 50 μ in diameter in an animal possessing a peduncle 2 mm long. Each cell contains a highly lobulated nucleus with a single nucleolus. There is an intracellular canal within each cell and this leads into the collecting duct. The secretion within the cell is aggregated close to the intracellular canal (Fig. 6); histochemical tests on this secretion confirm those of an earlier study on barnacle cement (Walker, 1970) which show it to be proteinaceous. Also in the cytoplasm there are distinct granules, 1μ in diameter, which stain intensely red with azan. Similar granules have been found in the cement cells of *Chelonibia testudinaria*, the turtle barnacle, and are thought to be lipofuscin granules (Walker, unpublished). There are in some cement cells of *O. mülleri* clear vacuoles, up to 5 μ in diameter. The cement cells of *L. anatifera* are rounded in shape, 56–112 μ in diameter (in a barnacle with a peduncle length of 4 mm), with spherical nuclei each containing 8–10 nucleoli. There is an extensive intracellular canal within each cell and this leads into a collecting duct. The region of the cytoplasm surrounding the intracellular canal is filled with secretion which as for *O. mülleri* reacts positively for proteins. Between the cement cells of *L. anatifera* there are many oblique muscle fibers (Fig. 7), which may aid the extrusion of secretion from the cells (see Lacombe, 1970).

Discussion

O. müllcri has been found on crabs which live in high salinity water, the crabs being either true oceanic crabs or spending part of their life cycle in the ocean. Pearse (1936), however, found an exception to this in *Uca minax* a crab which inhabits low salinity marshes. In the case of *C. sapidus*, the most common host of *O. müllcri* in Beaufort Inlet, the larval stages are spent in the ocean. As young crabs they migrate into low salinity water of estuaries, where they reach maturity after 1 year. Blue crabs mate in low salinity water, the mating taking place when the female is moulting for the final time (Judy and Dudley, 1970). The mated females then migrate back to high salinity water, where they spawn and where the larvae are released. Most males remain in the low salinity areas, only a few migrating back to the ocean with the females (Judy and Dudley, 1970). After the first spawn and hatch the females may re-enter the mouth of the estuary and spawn again, returning to the ocean for the second hatch. Finally, after spawning perhaps for a third time the females are thought to die in the ocean. Blue crabs rarely survive longer than 1 year after reaching maturity (Tagatz, 1968).

In trawls taken at Core Creek, in the Newport River upstream of the Beaufort Inlet where the salinity is 30‰ near the river bed only a single barnacle infested crab was taken, while at Adams Creek where the salinity close to the river bed was 16‰, *O. müllcri* was absent from all the blue crabs caught. These preliminary field observations help confirm Scarff's (1966) observations in the laboratory of the salinity tolerance of adult *O. müllcri*. She found that in water of salinity 20–40‰ the barnacles, which were left attached to pieces of excised gill, had open valves with the cirri extended. At 15‰ the valves were closed, but when the barnacles were returned to high salinity water 80% recovered after 24 hrs. At 10‰ valves were tightly closed and only 35% recovered after 24 hrs. At 5‰ the valves were tightly closed and none recovered. Salinity, therefore, appears to be a major factor limiting the distribution of *O. mülleri*.

DeTurk (1940) believed that the occurrence of *O. mülleri* was associated with the accumulation of debris in the branchial chambers of crabs, older blue crabs with large amounts of sand and other debris harboring the largest number of barnacles. He compared the infestation of dirty-gilled crabs, *L. emarginata, L. dubia* and *C. sapidus* with clean-gilled crabs, *O. ocellatus, P. spinimanus* and *C. ornatus* (see Table II), where few *O. mülleri* occur. *O. müllcri* settles on most crabs close inshore (Pilsbry, 1907) and therefore it is said to be a shallow water species (DeTurk, 1940; see Newman, 1967). Settlement is not host-specific and will take place on crabs of all age groups and sizes. However since juvenile crabs moult regularly, extruding the settled barnacles with the moult, the chance of observing *O. mülleri* on juvenile crabs is much less than on mature crabs. Although *O. mülleri* was found on many different crabs in Beaufort Inlet, *C. sapidus* is the most important host because it is the most common crab in this area (Judy and Dudley, 1971).

The respiratory current of the spiny lobster, *Puerulus sewelli,* was shown by Dinamani (1964) to affect the orientation of the cyprids of *Octolasmis stella* during initial settlement. They settled in such a position that following metamorphosis the young barnacle's cirral net was facing into the current. Specimens of *O. mülleri* were also found to be orientated to the respiratory current of the blue crab. Dinamani (1964) further explored the variation in certain features of the capitular valves of *O. stella;* he noted that the barnacles close to the outside of the branchial chambers of the lobster had armoured valves, whilst those barnacles better protected further in the branchial chamber had thinner, more delicate valves. Such differences due to situation were sought but not detected in *O. mülleri.*

In the relationship between the host crab and the epizoic barnacle most advantages lie with the barnacle. Once established in a branchial chamber it is protected, such protection allowing the reduction of capitular shell peduncular cuticle and musculature (*cf. Lepas anatifera*). A further advantage of being positioned in a branchial chamber is that there is a constant food supply brought in as suspended material in the crab's respiratory current. The presence of barnacles on the gills probably does not have any detrimental effects on the crab. However, bryozoans and nemerteans are regularly associated with barnacle infestations and all these epizoites would contribute greatly to the accumulation of debris on the gills and would consequently decrease the efficiency of the respiratory processes by impairing gill movements, reducing the amount of exposed gill surface and removing oxygen for their own needs.

The crabs main protection is ecdysis. After breeding the female blue crab has fulfilled her role in propagating the species and survival becomes less important. Moulting is terminated and she gradually becomes debilitated under epizoite attack.

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SUMMARY

1. *O. mülleri* was present on the gills of most crab species in Beaufort Inlet but not on *C. sapidus* further upriver indicating that salinity is probably a factor controlling the incidence of the barnacle.

2. The distribution of the barnacle on the individual gills of *C. sapidus* has been analyzed and the factors affecting this distribution discussed. The main factors are the cleaning action of the epipodites and the respiratory flow of the crab.

3. The barnacle settlement stage larva (cyprid) attaches to blue crab gills a short distance in from the gill margin. The orientation of the larva at settlement

is a response to the respiratory flow of the crab resulting in the cirral net of the young barnacle facing into the current.

4. The cement apparatus and internal stalk structures of *O. mülleri* and *Lepas anatifera* are compared.

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